Molecular Mechanisms underlying Dietary Modulation of Adult Hippocampal Neurogenesis

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Molecular Mechanisms underlying Dietary Modulation of Adult Hippocampal Neurogenesis

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Completing a PhD is the end of a very long, committed journey and it also marks the beginning of a new one. There are several people that helped me to make this sometimes seemingly impossible journey come to a happy end:

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Abstract

The studies described in this thesis explore the molecular mechanisms by which nutrition modulates Adult Hippocamapal Neurogenesis (AHN) using a human hippocampal progenitor cell line (HPC). AHN has been shown to be important for cognition and mood regulation. Interestingly, diet in the form of the Omega-3 fatty acids Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) is known to have beneficial effects on cognition and mood; these effects are hypothesised to be mediated via modulating AHN. My studies show that in an *in vitro* model of stress, EPA (10μM) and DHA (10μM) prevent the detrimental effects of Cortisol on proliferation and neurogenesis by increasing the percentage of dividing cells and neurogenesis while decreasing apoptosis mainly by promoting survival. Resveratrol (1μM), a stilbenoid present in the skin of red fruit, prevents the Cortisol induced changes by increasing the percentage of dividing cells and neurogenesis but has no effect on apoptosis. Intermittent fasting (IF), shown to promote AHN, increases the expression of *Klotho*, the longevity gene. Klotho over expression increases neurogenesis. Whereas Klotho knock down decreases neurogenesis by diminishing survival. To further investigate the molecular pathways downstream of Resveratrol as mimetic of IF and *Klotho* expression, I focused on PPARγ (Peroxisome proliferator-activated receptor) a nuclear receptor transcription factor which is known to activate Klotho transcription and is activated by Resveratrol. My results show that Resveratrol partly requires PPARγ to activate Klotho and that Klotho is partly required during proliferation for Resveratrol to exert its effect. Whereas, Resveratrol partly requires both Klotho and PPARγ to increase neurogenesis and PPARγ requires Klotho to increase the proportion of mature neurons. These experiments provide evidence that PPARγ and Klotho are two of the neurogenic effectors of Resveratrol.

This thesis provides for the first time evidence in a human *in vitro* model of neurogenesis and stress that EPA, DHA and Resveratrol can modulate neurogenesis and prevent its decrease induced by stress. This study also provides the first identification of Klotho and PPARγ as downstream effectors of Resveratrol on neurogenesis.

Publications arising from this thesis

Stangl,D.; Thuret,S.; Genes Nutr., 2009; Impact of diet on adult hippocampal neurogenesis

Oral communications of this PhD thesis

The Cambridge Neural Stem Cell Symposium, biannual meeting, Cambridge, U.K. (2011)

Cellular & Integrative Neuroscience, meeting, London, U.K. (2011)

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Adult Neurogenesis, abcam: Structure and Function, conference, Munich, Germany (2010)

International Society for Stem Cell Research (ISSCR), annual meeting, Barcelona, Spain (2009)

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List of Abbreviations

4-OHT	4-hydroxytamoxifen
AA	Arachidonic Acid
ACC	Acetyl-CoA carboxylase
ALA	α-Linolenic Acid
AMPK	5' Adenosine Monophosphate-activated Protein Kinase
BAEC	Bovine Aortic Endothelial Cells
BDNF	Brain-derived neurotrophic factor
bFGF	basic Fibroblast Growth Factor
bp	base pairs
BrdU	5’-Bromo - 2’ deoxyuridine
CaKMII	calcium-calmodulin-dependent protein kinase II
CD133	Prominin 1 or AC133
CORT	Cortisol
COX	Cyclooxygenase
CREB	**C**yclic adenosine monophosphate (cAMP) **R**esponse **E**lement binding protein
CSF	Cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
Dcx	Doublecortin

DHA	Docosahexaenoic Acid
Dlx2	distal-less homeobox 2
DNA	Deoxyribonucleic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eNOS	endothelial Nitric Oxide Synthase
EPA	Eicosapentaenoic Acid
ERK	Extracellular signal Regulated Kinases
GABA	*gamma*-Aminobutyric acid
HDL	High-density lipoprotein
HUVEC	Human Umbilical Vein Endothelial Cells
IGF1	Insulin/insulin-like Growth Factor-1
IGF-1R	IGF-1 Receptor
IL	Interleukin
IR	Insulin Receptor
JNK	c-Jun N-terminal Kinases
Ki67	“Kiel 67”
LA	Linoleic Acid
LDL	Low-Density Lipoprotein

LTP	Long Term Potentiation
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-Activated Protein Kinase
Mash1	Achaete Scute Complex-like 1 (ASCL1)
MCAO	Middle Cerebral Artery Occlusion
MDD	Major Depressive Disorder
mRNA	messenger Ribonucleic Acid
NAD+	Nicotine Adenine Dinucleotide
NDS	Normal Donkey Serum
NeuN	Neuronal Nuclei
NMDA	N-methyl-D-aspartate
NPC	Neural Progenitor Cell
NSC	Neural Stem Cells
PBS	Phosphate Buffered Saline
PC	Phophatidylcholine
PDE4	Phosphodiesterase type 4
PE	Phophatidylethanolamine
PFA	Paraformaldehyde

PGC-1α	**P**eroxisome proliferator-activated receptor **G**amma **C**oactivator 1-alpha
PS	Phosphatidylserine
PSA-NCAM^{+}	Poly-Sialylated Neural Cell Adhesion Molecule
qPCR	quantitative real-time Polymerase Chain Reaction
RSVL	Resveratrol
S100beta	S100 calcium binding protein B
SGZ	Subgranular Zone
SIR2	Silent Information Regulator 2
SIRT1	Silent mating type Information Regulation 2 homolog 1
SOX2	Sex determining region Y box 2
S-phase	Synthesis phase
SVZ	Subventricular Zone
TNF	Tumor Necrosis Factor
TRPV5	Transient Receptor Potential Vanilloid type isoform-5
VSMC	Vascular Smooth Muscle Cells

Chapter 1 Introduction

1.1 Neurogenesis in the adult brain

“Once development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centres, the nerve paths are something fixed, and immutable: everything may die, nothing may be regenerated” (Cajal, 1928).

The well known statement from Santiago Ramon y Cajal in 1928 had been taken for granted until Altman and Das (Altman and Das, 1965) provided the first evidence of postnatal hippocampal neurogenesis in rats using autoradiographic and histological methods. However, adult neurogenesis in the mammalian brain was not generally accepted until the early nineties when studies using Bromodeoxyuridine (BrdU) and retroviral vectors as markers for dividing cells confirmed the generation of new neurons in certain regions of the adult mammalian brain (Corotto et al., 1993; Luskin, 1993; Seki and Arai, 1993). Adult neurogenic niches have been consistently found in two restricted areas: the subventricular zone (SVZ) of the lateral ventricles (Doetsch and Alvarez-Buylla, 1996) and in the subgranular zone (SGZ) of the dentate gyrus (DG) (Kempermann and Gage, 2000). In these regions neural stem cells (NSCs) and the more specified neural progenitor cells (NPCs) have been shown to give rise to new neurons in various species (Altman and Das, 1965; Kaplan and Hinds, 1977; Bayer et al., 1982; Kaplan and Bell, 1984; Luskin, 1993; Doetsch and Alvarez-Buylla, 1996; Sanai et al., 2011) including humans (Eriksson et al., 1998).

Neurogenesis in the adult brain is divided into specific steps: proliferation and survival of NPCs and NSCs, as well as survival, differentiation, maturation and integration of early and mature neurons. Adult NSCs and adult NPCs are self-

replicating, multipotent cells that can differentiate into multiple types of neural cells, including neurons, astrocytes and oligodendrocytes (Gage, 2000; Taupin and Gage, 2002). See Figure 1-1. NSCs have the potential to divide indefinitely and give rise to NPCs. NPCs have a limited capacity to self-replicate and differentiate into target cells after a restricted number of divisions (Taupin and Gage, 2002; Lucassen et al., 2009).

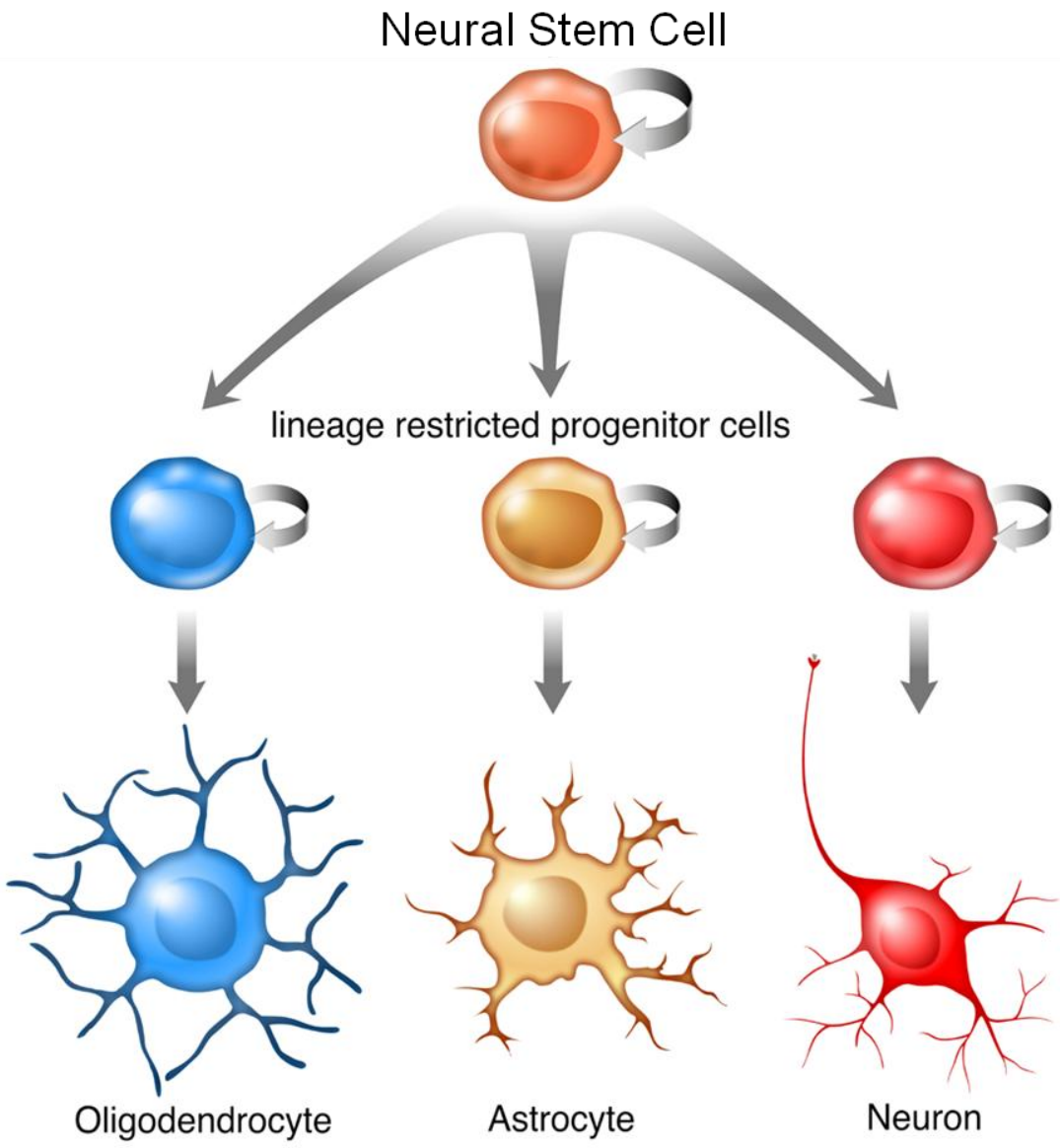


Figure 1-1 Neurogenesis is the birth of new neurons
Neural stem cells are defined as cells that have the ability to self-renew and to give rise to the three major cell types of the mammalian CNS: neurons, astrocytes, and Oligodendrocytes. Credit: J. Simons, Illustrator at The Salk Institute for Biological Studies.

Two types of NSCs have been identified in the SVZ and the SGZ that are distinguishable by their morphology, proliferating behaviour and marker expression (Zhao et al., 2008; Mu et al., 2010): In the SVZ type B cells are GFAP and CD133 positive radial glia-like progenitor cells. These slowly dividing cells generate the more rapidly dividing type C cells; transit-amplifying progenitor cells with very short or no processes that are positive for the marker Dlx2, Mash1 and EGFR. The majority of those will turn into type A cells: Dcx and PSA-NCAM expressing neuroblasts that migrate via the rostral migratory stream into the olfactory bulb where they differentiate into GABAergic and dopaminergic interneurons. In the SGZ the radial cells or type 1 cells are positive for the marker GFAP, SOX2 and Nestin. This population of quiescent NSCs might give rise to type 2 cells; actively self-replicating nonradial progenitor cells that are SOX2 and Nestin positive but GFAP negative and might generate Dcx positive neuroblasts that eventually differentiate into glutamatergic dentate granule cells (Figure 1-2).

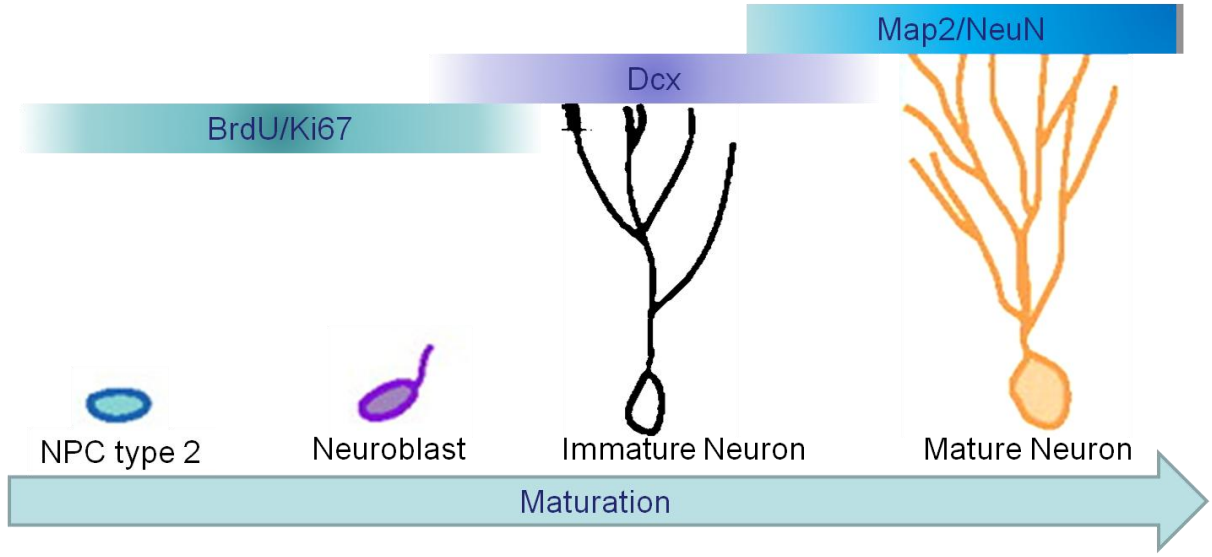


Figure 1-2 Overview of the maturation of NPC to mature neurons and the markers they express.

Many of the newly generated neurons die within two weeks, however the surviving ones become fully integrated and functional (Kempermann et al., 2003). In Figure 1-3 it is shown how newborn neurons in the hippocampus migrate only a short distance into the granule cell layer. Within three weeks they send axons to the CA3 region and the hilus to form functional synapses with interneurons from the hilus and neurons from the CA3 (Chan et al., 2008). The new neurons start to receive synaptic inputs from the cortex and fire action potentials themselves (van Praag et al., 2002). NPCs from the SVZ in turn migrate a greater distance via the rostral migratory stream (RMS) into the olfactory bulb to become interneurons (Doetsch and Alvarez-Buylla, 1996).

It is still not perfectly understood why neurogenesis is restricted to the hippocampus and the SVZ considering that NPCs have been isolated from many CNS regions. However, several transplantation experiments (Suhonen et al., 1996; Shihabuddin et al., 2000; Seidenfaden et al., 2006) have shown that the fate of transplanted NSCs is determined by the microenvironment of the graft side. For example, NPC from the spinal cord can normally not give rise to neurons. However, once transplanted in the DG or SVZ, spinal cord NPCs can generate neurons (Shihabuddin et al., 2000). This demonstrates the importance of the microenvironment of the neurogenic niches. Where and how neurogenesis occurs is tightly orchestrated by a multitude of cell intrinsic and extrinsic factors. Intrinsic factors include CREB (Giachino et al., 2005) and NeuroD1 (Gao et al., 2009), whereas extrinsic factors include BDNF (Bath et al., 2008) and GABA (Gascon et al., 2006) or Glutamate (Platel et al., 2010). For a detailed review see (Mu et al., 2010).

It has long been understood that AHN is associated with learning and memory as well as mood and can be influenced internally via molecular and epigenetic factors as well as external factors such as environment, stress, learning, exercise, aging and diet (Figure 1-4). For a review see (An et al., 2008). A decline in neurogenesis in the SGZ during ageing is associated with increasingly impaired cognitive functions, which can be ameliorated by external events with systemic impact, such as voluntary running (van Praag et al., 2005).

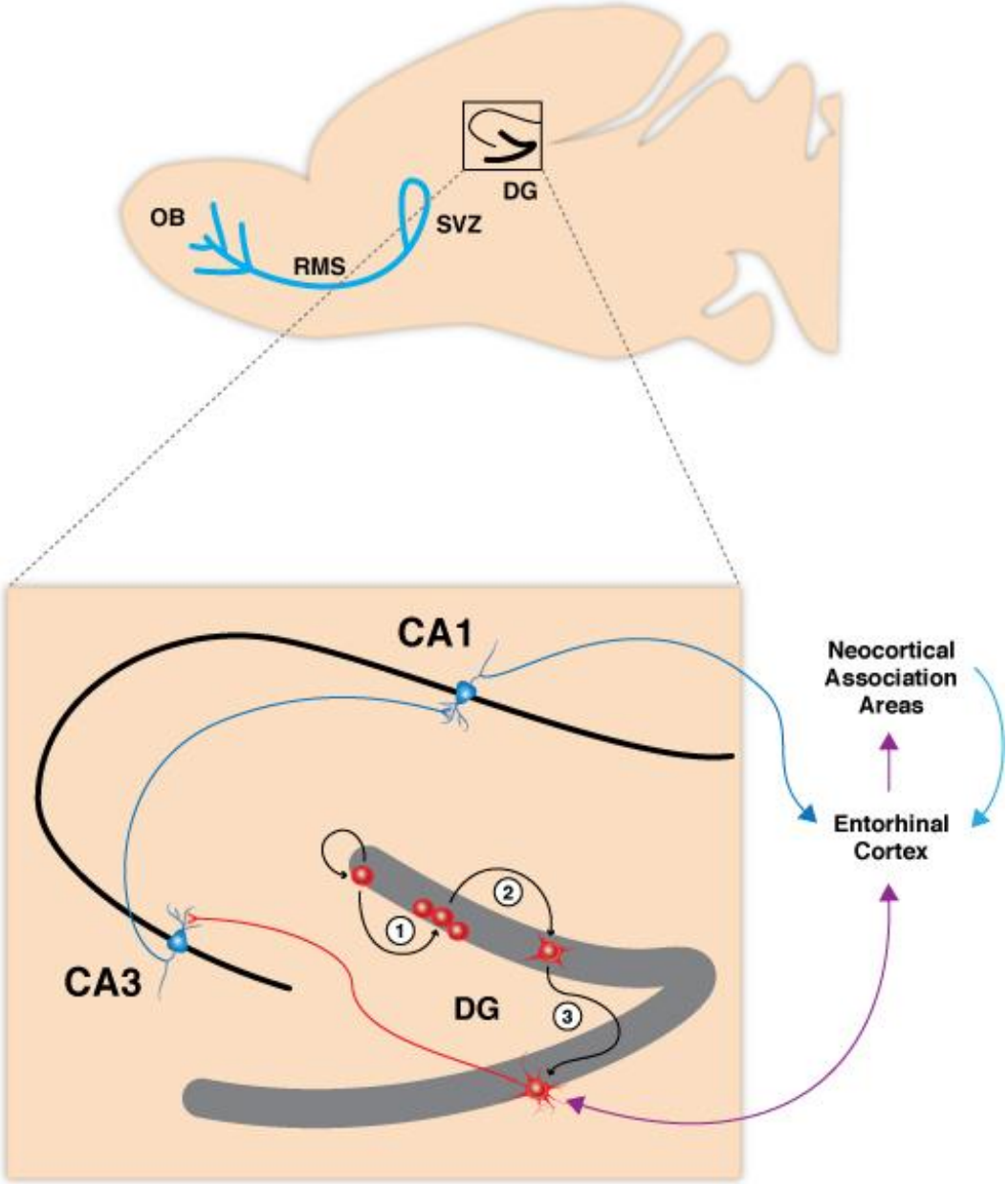


Figure 1-3 Schematic representation of the sagittal view of a rodent brain highlighting the two neurogenic zones of the adult mammalian brain: The subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the dentate gyrus (DG) in the hippocampus. Neurons generated in the SVZ migrate through the rostral migratory stream (RMS) and are incorporated into the olfactory bulb. The hippocampal region contained in the black square is enlarged showing (1) neural progenitor cells in the in the subgranular zone of the dentate gyrus proliferating, (2) migrating as neuroblasts into the granule cell layer and (3) maturing into new granule neurons. They integrate into the hippocampal circuitry by receiving inputs from the entorhinal cortex, and extend projections into the CA3. (Stangl and Thuret, 2009)

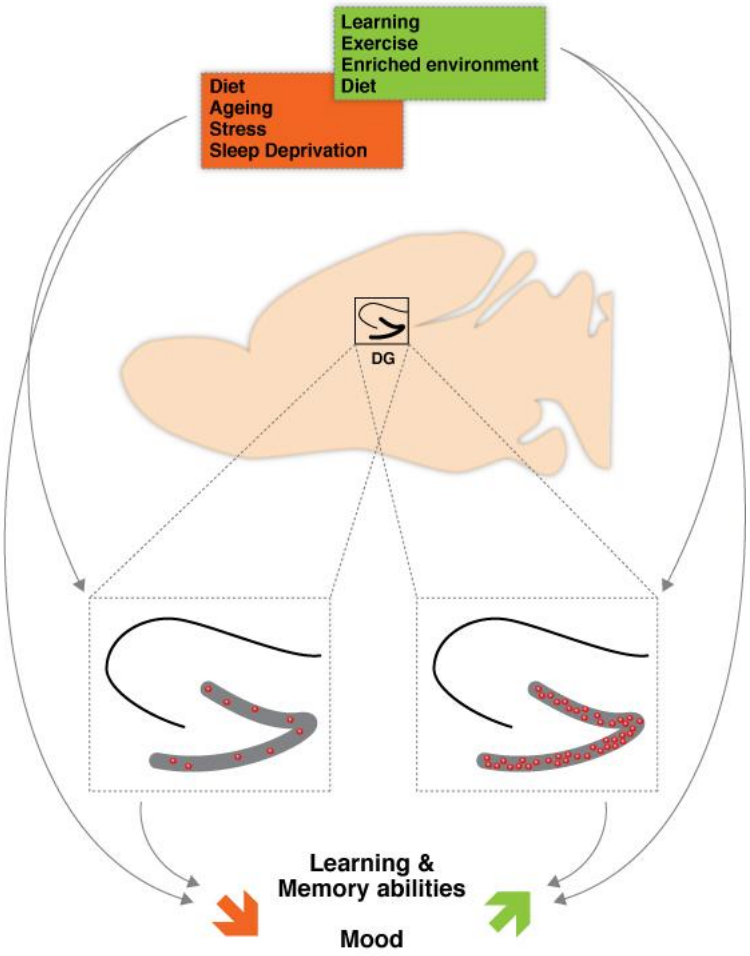


Figure 1-4 Overview of physiological and environmental modulation of Adult Hippocampal Neurogenesis
and its impact on Learning & Memory abilities and Mood. The doyted squares contain the enlarged hippocampus. The red dots symbolize newborn neurons in the dentate gyrus. (Stangl and Thuret, 2009)

1.2 Functionality of adult hippocampal neurogenesis

This thesis is set out to explore the impact of external factors such as diet on AHN. The hippocampus plays an important role in learning, memory and mood, which will be explored in detail in sections 1.2.1 and 1.2.2. As described in Figure 1-4 hippocampal neurogenesis can be modulated by multiple environmental stimuli and is considered to be necessary for optimisation and adaption of hippocampal function to environmental changes by experience Reviewed in (Glasper et al., 2012). Briefly, under optimal environmental conditions neurogenesis and exploratory behaviour are increased to enable the

development of greater cognitive function and spatial navigation skills. In turn, under prolonged adverse and potentially life threatening conditions, hippocampal neurogenesis is decreased which amongst other effects leads to inhibited exploratory behaviour and increased anxiety to maximise the likelihood of survival by staying out of danger (Glasper et al., 2012). These consequences might explain the important implications AHN has on improved learning and memory or developing anxiety and depression like behaviour.

The focus of this thesis is the modulation of AHN by certain dietary components described in section 1.3 and the possible effect on learning, memory (Section 1.2.1) and depressive symptoms (Section 1.2.2).

1.2.1 Learning and Memory

A multitude of studies has been conducted to explore the relation between neurogenesis in the dentate gyrus and cognitive ability. They aimed to find an answer on whether and how neurogenesis in the dentate gyrus is involved in acquiring, storing, short or long-term retention and retrieval of spatial memory or object/place recognition, reviewed in (Aimone et al., 2010a; Koehl and Abrous, 2011). Due to several confounding factors, such as variations in mouse strains, ablation technique and experimental settings, these studies produced inconsistent results. However, correlative evidence, ablation studies and computational modelling support the putative function of AHN in various forms of hippocampal dependent learning and memory (An et al., 2008; Aimone et al., 2009; Aimone et al., 2010a). New neurons in the dentate gyrus are said to be involved in memory processing during spatial learning, associative memory and pattern separation (Reviewed in (Stangl and Thuret, 2009). The level of hippocampal neurogenesis and the performance in hippocampal-dependent

learning tasks has been shown to positively correlate in various mouse models (Kempermann and Gage, 2002; Thuret et al., 2009). Further, it has been shown that changes in neurogenesis induced by the environment correlate with performance in hippocampal-dependent learning tasks (Reviewed in (Stangl and Thuret, 2009). Interestingly, not only the generation and integration of new cells plays an important role during learning and the formation of memory but apoptosis as well. Blocking apoptosis prevents the survival of dentate granule cells and proliferation of NPC, leading to an impaired performance in spatial learning and memory. This suggests that learning is a dynamic process during which dentate granule cells are selectively added and removed from the neuronal network (Dupret et al., 2007).

Many genetic and environmental factors that affect AHN cause corresponding changes in cognitive performance. Voluntary running (van Praag et al., 1999; Spanier et al., 2009; Aimone et al., 2010a, b; Deng et al., 2010; Yu et al., 2010; Marlatt et al., 2012) and enriched environment (Kempermann et al., 1997b, a; Kuhn et al., 1997; Bruel-Jungerman et al., 2005; Tashiro et al., 2007; Rippe et al., 2010; Chiu et al., 2012; German et al., 2012; Liu et al., 2012) lead to an increased AHN and an improved performance in spatial and long term memory tasks. In contrast, AHN decreases with age and elderly animals display impaired learning and memory abilities (Klempin and Kempermann, 2007; Villeda et al., 2011). Interestingly, age-dependent blood borne factors from old mice negatively affect adult neurogenesis and associated behaviour such as spatial learning, and fear conditioning as shown in a heterochronic parabiosis experiment joining young and old mice (Villeda et al., 2011).

When investigating the role of adult born neurons we have to question whether their contribution to memory processing in the DG is unique and distinct compared to that from neurons born during development. Kee et al. showed for the first time that adult born neurons were preferentially recruited by spatial learning tasks using immediate early genes (IEG) to image the activity of adult-born neurons (Kee et al., 2007). However, the same team showed that developmentally born and adult born granule cells are integrated into hippocampal memory networks at similar rates. Their data further suggest a functional equivalence between DG cells generated at different developmental stages (Stone et al., 2011). Before the role of granule neurons generated at different developmental stages can be definitely regarded as equivalent these results need confirmation and further investigation.

1.2.2 Depression

Worldwide, major depressive disorder (MDD) is estimated to become the second leading cause of disease burden by the year 2030 after HIV (Mathers and Loncar, 2006). Depression is defined mainly by diagnostic criteria formulated by the Diagnostic and Statistic Manual IV (DSM-IV) published by the American Psychiatric Association (2000). Symptoms include low self esteem, lack of interest, sleep disturbance, eating disturbance, poor concentration and suicidal thoughts. The heterogeneity of the disease suggests that multiple different neural mechanisms and distinct brain regions may underlie its aetiology (Manji et al., 2001;Nestler et al., 2002;Warner-Schmidt and Duman, 2006). In human imaging and post mortem studies the prefrontal and cingulate cortex, hippocampus, striatum, amygdala and thalamus have been identified as brain areas that are associated with depression (Drevets, 2001; Liotti and Mayberg, 2001; Manji et al., 2001; Nestler et al., 2002). The highly

intercommunicative connections these brain regions form are implicated in depression (Manji et al., 2001; Nestler et al., 2002). To shed further light on the processes during depression and improve treatment, the cellular mechanisms within these regions need to be identified.

One of the theories trying to explain the mechanism of depression is the neurogenesis hypothesis. It claims that the decrease in newborn dentate granule cells in the dentate gyrus is associated with the pathophysiology of depression (Becker and Wojtowicz, 2007; Vollmayr et al., 2007). This hypothesis is further supported by the fact that antidepressant treatments enhanced neurogenesis (Gould et al., 1997). However, while decreasing neurogenesis alone is not sufficient to initiate a depression-like phenotype, neurogenesis in the hippocampus is required for antidepressants to elicit some of their positive behavioural effects. Reviewed in (Samuels and Hen, 2011).

Neurogenesis can be reduced by stress, a causal factor in the pathogenesis of major depression. Upon exposure to a stressor the hypothalamic-pituitary-adrenal axis (HPA) releases high levels of endogenous glucocorticoid hormones, like the corticosterone Cortisol. This reaction, controlled by the hippocampus, provides the body with energy, increases focus and attention and helps to respond to immediate potentially life-threatening situations (Pariante, 2009). However, chronically elevated levels of glucocorticoids can lead to disturbances in reproductive functions, metabolic functions, brain electrophysiological properties, and depressive symptoms, reviewed in (Pariante, 2009). Chronic exposure to stressful conditions is a commonly used model for depression in laboratory animal and has been shown to reduce AHN. (For a review see (Warner-Schmidt and Duman, 2006)). Furthermore, AHN is

reduced in animal models of depression (Dranovsky and Hen, 2006). Indeed, acute and chronic treatment with corticosterone decreases cell proliferation (Gould et al., 1992; Murray et al., 2008; Cao et al., 2009; Colman et al., 2009; David et al., 2009; Spencer et al., 2009; Vafeiadou et al., 2009; Kennedy et al., 2010; Thurston et al., 2010) whereas adrenalectomy increases AHN. Additionally, glucocorticoids inhibit the proliferation, differentiation and survival of new cells within the hippocampus (Wong and Herbert, 2004). In this thesis an *in vitro* model of stress using Cortisol (Section 2.1.5.1) (Anacker et al., 2011) is employed to test the potential preventive properties of dietary components (Section 1.3) against stressed-induced decreased neurogenesis.

Pharmacological treatment for depression with common antidepressant drugs, such as selective serotonin reuptake inhibitors (SSRIs) changes metabolic activity in the prefrontal cortex and the hippocampus (Mayberg et al., 2000; Kennedy et al., 2001; Seminowicz et al., 2004). Antidepressant treatment takes several weeks to show an effect in human; that delay suggests that structural changes in neural circuitry, such as adult neurogenesis, are involved in eliciting its positive effect (Duman et al., 1999; Duman and Monteggia, 2006; Warner-Schmidt and Duman, 2006; Sahay and Hen, 2008). Chronic administration of antidepressants, such as SSRIs and monoamine oxidase inhibitors, increases AHN and accelerates the maturation of young neurons in the DG (Malberg et al., 2000; Manev et al., 2001; Santarelli et al., 2003; Wang et al., 2008). Neurogenesis can also be increased by acute electroconvulsive therapy (ECT) (Malberg et al., 2000; Scott et al., 2000; Manev et al., 2001; Wang et al., 2008). Additionally, the effect of stress on AHN can be reversed by various antidepressants (Dranovsky and Hen, 2006; Surget et al., 2008). Importantly, these enhancing effects are restricted to the hippocampus and do not affect cell

proliferation in the SVZ, an area with no emotional behavioural relevance (Encinas et al., 2006). Finally, in several animal models of depression, disruption of AHN blocks the behavioural efficacy of antidepressants (i.e. Santarelli et al., 2003).

Not only environmental factors such as running, exercise and environmental enrichment (van Praag et al., 1999; Bjornebekk et al., 2005; van Praag et al., 2005; Chiu et al., 2012; Marlatt et al., 2012) have been associated with an antidepressant effect possibly via enhancing AHN, but various components of our daily diet have now been correlated to impact on neurogenesis and also to improve cognition and depressive behaviour.

1.3 Dietary influences on AHN

Understanding the molecular mechanisms by which diet influences adult hippocampal neurogenesis could help in preventing and treating cognitive and mood decline and moreover lead to new therapeutic targets.

The four different levels on which dietary modulation of AHN can take place shown in Figure 1-5 are: Calorie intake, meal frequency, meal texture and meal content. These four parameters modulate AHN as well as cognitive performance and mood as shown in animal models and intervention or epidemiological studies in humans (Reviewed in (Stangl and Thuret, 2009). Caloric intake and meal frequency in the form of dietary restriction (DR) and intermittent fasting (IF) will be explained in section 1.3.3 and 1.3.4. Regarding meal content, nutrients such as Omega-3 fatty acids (Omega-3s) (Section 1.3.1) and flavonoids (Section 1.3.2) have been shown to impact on AHN, cognition and depression.

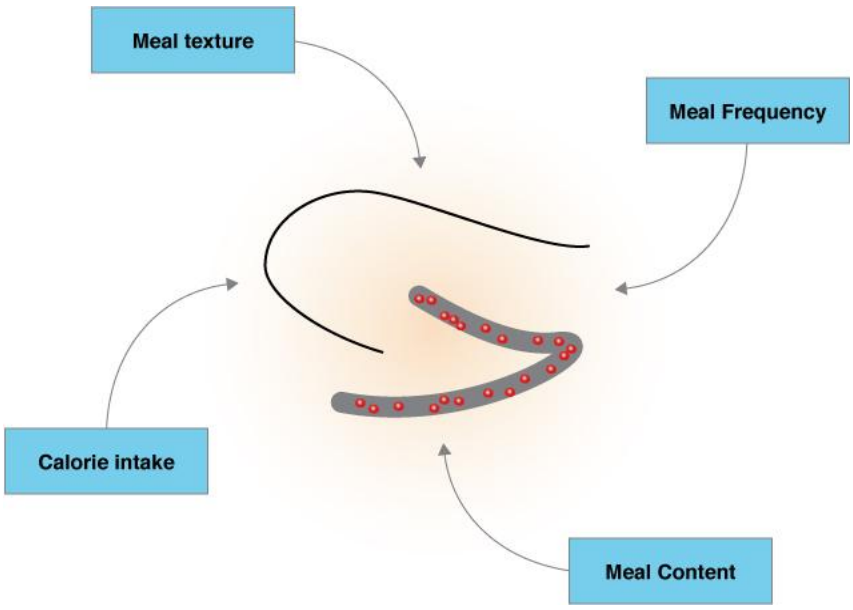


Figure 1-5 Overview of the impact of diet on adult hippocampal neurogenesis. The red dots symbolize newborn neurons in the dentate gyrus of the hippocampus. (Stangl and Thuret, 2009)

1.3.1 Omega-3 and omega-6 fatty acids

One of the dietary ingredients that are highly discussed at present in association with depression and learning and memory abilities are Omega-3 fatty acids (Omega-3s).

The Omega-3s, α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are nutritionally important and essential for maintaining a normal neurological function. Omega-3s cannot be synthesized by mammals and need to be supplied by diet. Common sources of Omega-3s include fish oils and plant oils such as flaxseed oil, algal oil and hemp seed oil.

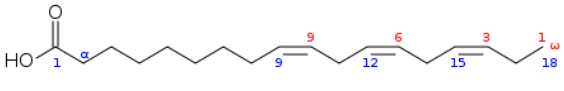
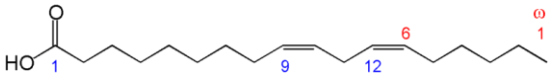
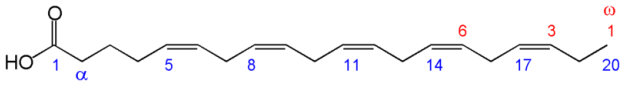
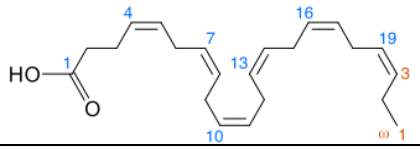
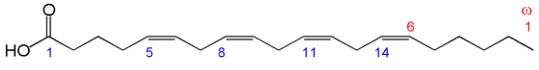
Essential fatty acids (short-chain fatty acids)	
α-Linolenic Acid (ALA) (18:3 n-**3**)	
Linoleic acid (LA) (18:2 n-**6**)	
Conditionally essential fatty acids (long-chain fatty acids)	
Eicosapentaenoic Acid (EPA) (20:5 n-**3**)	
Docosahexaenoic Acid (DHA) (22:6 n-**3**)	
Arachidonic Acid (AA) (20:4 n-**6**)	

Table 1-1 Omega-3, Omega-6 fatty acids
Overview of essential and conditionally essential Omega-3 and Omega-6 fatty acids. Nomenclature: **18:3 n-3**: 18 is the number of C atoms, 3 is the number of C-C double bonds, n-3 denotes the position of first double bond at the third C atom counting from the omega end (red numbers); indicating an Omega-3 fatty acid. The blue numbers are the counting used by chemists and start at the carbonyl end.

In Table 1-1 the important essential and conditionally essential fatty acids are listed. True essential fatty acids are the Omega-3 fatty acids α-Linolenic acid (ALA) and the Omega-6 fatty acid Linoleic acid (LA) (Burr and Burr, 1930). The conditionally essential Omega-3s EPA and DHA are long-chain fatty acids that are not contained in plants; however they can be formed out of the short-chain fatty acid ALA in mammals, although very little ALA is converted to DHA in the adult human compared to during development (Innis, 2007). This ability is limited by the capacity of the enzyme Δ6 desaturase over which the Omega-6 and the Omega-3 pathway compete (Portolesi et al., 2007). The pathway for the biosynthesis of EPA and DHA is explained in Figure 1-6. Omega-3s are widely accepted to have beneficial effects on the brain and associated behaviour, whereas Omega-6s, such as Arachidonic acid (AA), increase the production of proinflammatory cytokines (Maes and Smith, 1998). Moreover, in depressed patients a higher Omega-6 to Omega-3 ratio correlated with higher TNF-alpha, IL-6, and IL-6 soluble receptor levels, suggesting that diets with high Omega-6 to Omega-3 ratios may enhance the risk for both depression and inflammatory diseases (Kiecolt-Glaser et al., 2007). The recommended ratio of Omega-6 to Omega-3 is 4:1 (Hibbeln et al., 2006), however, the actual ratio in the average diet of the western world is 30:1 (Chang et al., 2005). Omega-6s and Omega-3s are not inter-convertible in the human body. This imbalance might be a target for possible dietary intervention to improve learning and memory and depressive symptoms.

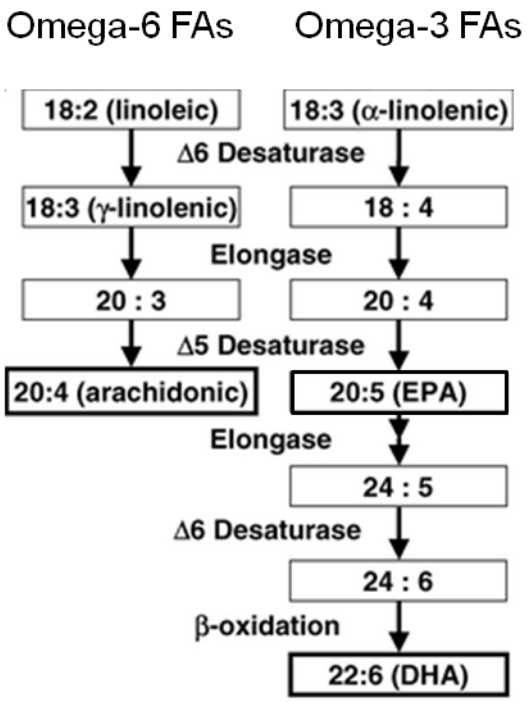


Figure 1-6 Pathway for Omega-3 and Omega-6 fatty acid biosynthesis
For the first three steps of their biosynthesis long-chain Omega-3 and Omega-6 fatty acids share the same enzymes: Δ6 desaturase, Δ5 desaturase. For the conversion of EPA to DHA Δ6 desaturase is used again. EPA: Eicosapentaenoic Acid, DHA: Docosahexaenoic Acid. Adopted from (Nakamura and Nara, 2004)

The dry weight of the mammalian brain consists to 50% of lipids, mainly phospholipids, of which DHA and AA are the main polyunsaturated fatty acids (PUFA) (Svennerholm, 1968; Sastry, 1985). DHA is predominately found in the phosphatidylethanolamine (PE) and the phosphatidylserine (PS) fraction which together with phosphatidylcholine (PC) constitute the major building block of neuronal membranes (Rapoport, 2001). The majority of DHA gets accumulated into the brain perinatally during development up to the second year after birth in humans (Martinez, 1992). Omega-3s play crucial roles in the development (Makrides et al., 2010) and function of the central nervous system (Crupi et al., 2011). They are critical for healthy brain development and function because of their roles in membrane structure and cytokine regulation (Innis, 2007; Gupta et al., 2012). However, phospholipid concentrations decline in the hippocampus

during aging (Delion et al., 1997) and neurodegenerative diseases such as Alzheimer's disease (AD) (Soderberg et al., 1991). Further, a deficiency of hippocampal Omega-3s is associated with a decrease in learning and memory abilities in rodents and a decline in memory in AD patients (Tully et al., 2003; Chung et al., 2008). However, the symptoms of an Omega-3 deficit can be reversed with a supplemented diet. In an animal model of adult immune stressed mice a diet enriched with PUFA decreased hippocampal microglia activation and increased neurogenesis in the hippocampus leading to normal long-term potentiation (LTP) (Crupi et al., 2011). One possible mechanism of how PUFA exert their positive action is their impact on the protein localisation in lipid rafts. DHA concentrations influence the properties and organisation of proteins in the cell membrane, affecting membrane fluidity (Langelier et al., 2010). Overall, increased DHA levels have been shown to promote neurite outgrowth, neuronal differentiation and synaptogenesis during development (Calderon and Kim, 2004; Cao et al., 2009; Adijiang and Niwa, 2010).

1.3.1.1 Omega-3s and learning and memory

Low intake of Omega-3s is associated with several forms of cognitive decline in the elderly (Freemantle et al., 2006) whereas a diet rich in Omega-3s is associated with a prevention of cognitive decline (van Gelder et al., 2007). Interestingly, rodents deficient for Omega-3s have shown impaired performance in spatial memory tasks that could be rescued with dietary intake of Omega-3s (Fedorova and Salem, Jr., 2006). In DHA deficient rats, the Omega-3 levels in the hippocampus could be rescued with a DHA supplemented diet and subsequently the performance in learning and memory tasks improved (Chung et al., 2008). Furthermore, Omega-3 supplementation during brain development, as well as during adulthood increased DHA levels of the

hippocampus and improved learning and memory abilities (Chung et al., 2008). It has been suggested that Omega-3s sustain learning and memory performance by reinforcing synaptic plasticity. Several *in vivo* experiments or *in vitro* set ups using rodent brain slices showed that a diet sufficient in Omega-3s can maintain or rescue LTP and enhance dendritic spine formation. DHA affects synaptic transmission in mammalian brain and increases the levels of phosphatides and of specific presynaptic or postsynaptic proteins in the brain cells of rodents receiving DHA (Cansev et al., 2008). Further DHA enhanced induced neurite growth, synaptogenesis, synapsin, and glutamate receptor expression, and glutamatergic synaptic function (Cao et al., 2009). Together this may support the role of DHA in important cellular aspects related to hippocampus-related cognitive function. Figure 1-7 shows an overview of the implications of Omega-3s during development (1.) and maintenance (2.) of learning and memory as reviewed in (Hui-Min, 2010).

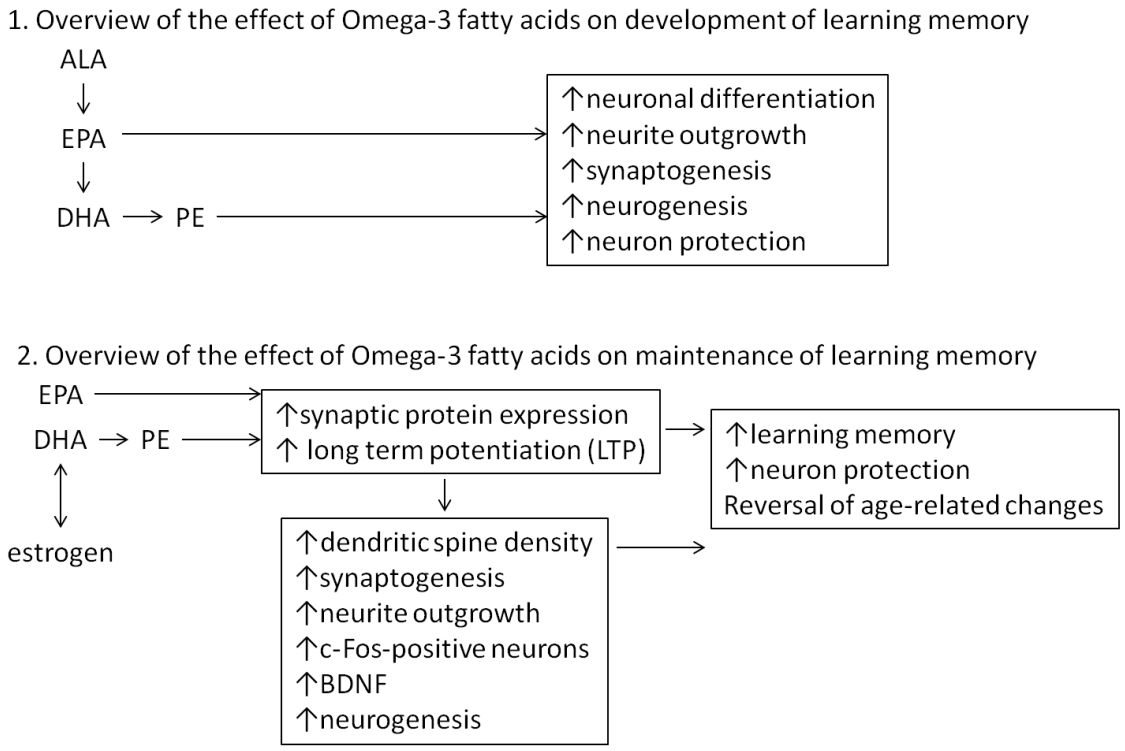


Figure 1-7 Overview of the effects of Omega-3s on the development (1.) and maintenance (2.) of learning memory performance.
(1.) Overview of effects on the development of learning and memory. The developing brain can carry out limited DHA biosynthesis from ALA and incorporate it into phosphatidylethanolamine (PE). Omega-3 signalling is involved in brain development. (2.) Overview of effects on maintenance of learning memory. The mature brain can take up DHA and incorporate it into the PE fractions of neuronal membranes, where Omega-3 signalling strengthens synaptic plasticity, increase neuron protection and reverse age-related changes. Interactions between the effects of DHA and oestrogen are possible. Adapted from (Hui-Min, 2010).

1.3.1.2 Omega-3s and Depression

Moreover Omega-3s have been shown to be beneficial in depression. Clinical research has shown that Omega-3 concentrations are lower in patients with depression (Logan, 2004), and Omega-3 supplementation has even emerged as a treatment for depression (Frangou et al., 2006; Jazayeri et al., 2008). In epidemiologic studies a diet high in Omega-3s is associated with a decreased risk of major depression, perinatal depression and bipolar disorder (Noaghiul and Hibbeln, 2003; Hibbeln, 2009). Trials supplementing PUFAs or comparing the amount of fish consumption generally show an improvement in depressive symptoms (Hibbeln, 1998; Tiemeier et al., 2003; Adams et al., 2008; Jazayeri et

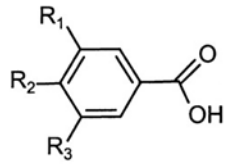
al., 2008); although the trials up to date are difficult to compare as neither the dose range (1-9 g/d) nor the ratio of EPA to DHA or their separate administration are consistent. Reviewed in (Perica and Delas, 2011). All together, we lack data from adequately powered control trials in human to be able to tell whether Omega-3 supplements are sufficient as a monotherapy in the treatment of major depressive disorder (MDD). Further reduced Omega-3 levels in the brain of depressed patients are not only owing to an insufficient supply but also due to interaction between diet and metabolism (Otten et al., 1997; Keelan et al., 2000). Some studies report a significant decrease of Omega-3s in depressed patients whereas Omega-6s levels hardly change (Maes et al., 1996; Maes et al., 1999; De Vriese et al., 2003; Tiemeier et al., 2003; Frasure-Smith et al., 2004) or are even increased (Maes and Smith, 1998). The Omega-6 fatty acid arachidonic acid increases the production of proinflammatory cytokines, operating as precursors of the proinflammatory eicosanoids of the prostaglandin 2-series (Maes and Smith, 1998). Furthermore, in patients with MDD higher Omega-6: Omega-3 ratio correlated with higher levels of the proinflammatory markers TNF-alpha, IL-6, and IL-6 soluble receptor. This suggests that diets with high Omega-6 to Omega-3 ratios may enhance the risk for both depression and inflammatory diseases (Kiecolt-Glaser et al., 2007). Interestingly, a diet high in Omega-6s and saturated fats even impacts on future generations through the exposure of the embryo in utero to the content of the maternal diet. This exposure affects the risk for obesity, neurocognitive function via immune response dysregulation and proinflammatory responses in the hippocampus leading to elevated levels of anxiety and spatial learning deficits (Bilbo and Tsang, 2010; Massiera et al., 2010). For a review of the effects of Omega-6s see (Simopoulos, 2011).

The mechanisms underlying these positive as well as negative behavioural effects are currently unknown. However it is likely that the positive effects on learning, memory (Hooijmans et al., 2009;Petursdottir et al., 2008) and mood (Appleton et al., 2006) are mediated by increased AHN as shown in animals fed with a diet rich in the Omega-3 fatty acid Docosahexaenoic acid (Kawakita et al., 2006). Similar positive results for cognition were achieved using a diet enriched in polyphenols and PUFA in an AD mouse model (Fernández-Fernández et al., 2012).

1.3.2 Polyphenols

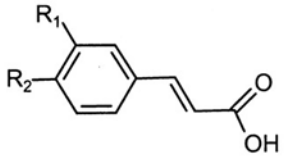
Polyphenols are secondary metabolites of plants and are generally involved in defence against ultraviolet radiation or pathogens, such as bacteria and fungi. These molecules are derived from phenylalanine and contain an aromatic phenol ring with a reactive hydroxyl group. Some dietary sources for polyphenols are citrus, tea, ginko, grape, cocoa and blueberries. They consist of several subclasses including phenolic acids, flavonoids, stilbenes and lignans (Figure 1-8). The biggest group are the flavonoids which are subdivided in flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanoles.

Hydroxybenzoic acids



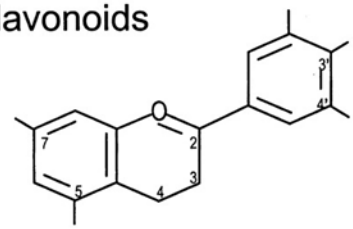
$R_1 = R_2 = OH$, $R_3 = H$: Protocatechuic acid
$R_1 = R_2 = R_3 = OH$: Gallic acid

Hydroxycinnamic acids



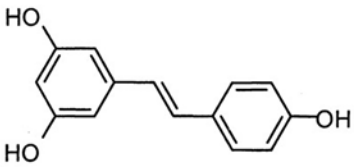
$R_1 = OH$: Coumaric acid
$R_1 = R_2 = OH$: Caffeic acid
$R_1 = OCH_3$, $R_2 = OH$: Ferulic acid

Flavonoids



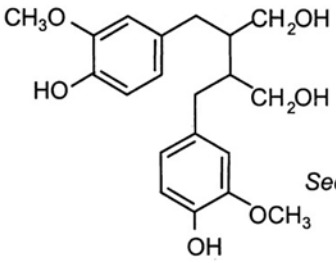
See figure 1-9 for a Flavonoid overview

Stilbenes



Resveratrol

Lignans



Secoisolariciresinol

Figure 1-8 Overview of the chemical structure of polyphenols. Adapted from (Manach et al., 2004)

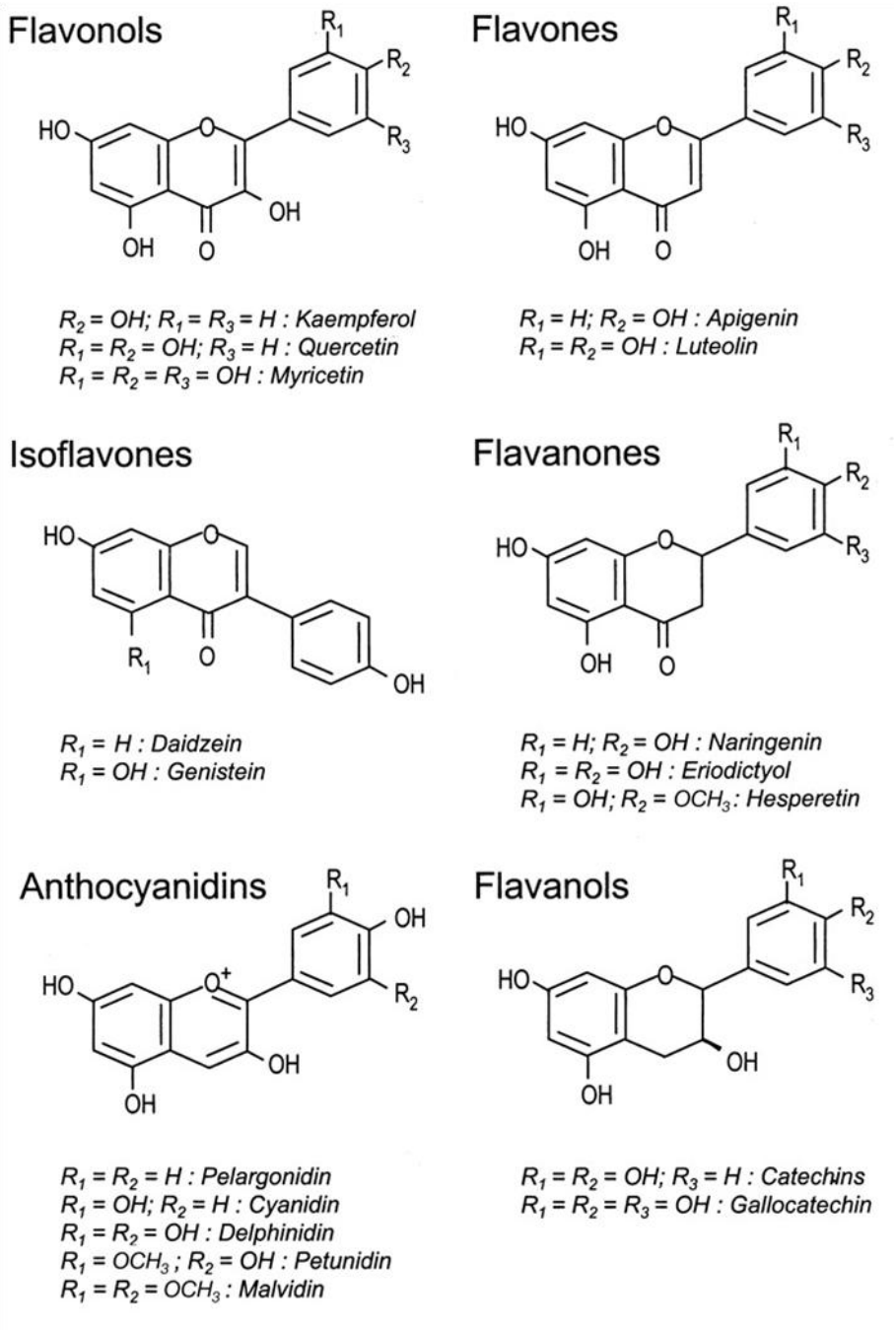


Figure 1-9 Overview of the chemical structure of flavonoids. Adapted from (Manach et al., 2004).

Polyphenols are said to have neuroprotective effects through interaction with neuronal signalling pathways, inhibition of neuroinflammation and impact on the vascular system, reviewed in (Spencer, 2010a). For example, studies in humans have shown that polyphenols-rich dark chocolate decreases blood pressure (Taubert et al., 2003). Further studies have reported that flavanol-rich cocoa improves vasodilatation (Heiss et al., 2003; Taubert et al., 2003). Also wine, grape juice and black tea have been shown to have a vasodilatatory effect (Kukekov et al., 1999; Hirata et al., 2004; Janszky et al., 2005). Flavanols in cocoa have been shown to improve cerebrovascular blood flow (Fisher et al., 2006; Francis et al., 2006) and increased blood flow in the brain is known to facilitate AHN (Ruitenberg et al., 2005; Tiehuis et al., 2008). Additionally, fMRI studies have shown that cerebral blood flow is decreased in patients with dementia (Nagahama et al., 2003; Ruitenberg et al., 2005) whereas it is increased shortly after flavanol consumption (Fisher et al., 2006; Wang et al., 2008). Furthermore, it has been demonstrated that flavonoids inhibit neuroinflammatory effects. Interestingly, neuroinflammation plays an important role in Alzheimer's and Parkinson's disease (McGeer and McGeer, 2003; Hirsch et al., 2005) as well as brain injuries such as stroke (Zheng et al., 2003). Flavonoid-rich blueberry extract decreased nitric oxide, IL-1β and TNFα production (Lau et al., 2007). The flavonol quercetin (Chen et al., 2005), the flavone wogonin (Lee et al., 2003) and the flavanone naringenin (Vafeiadou et al., 2009) also reduced nitric oxide and TNFα levels, factors that are largely involved in microglial and astrocytic mediated neuroinflammatory effects.

Flavonoids further impact on synaptic plasticity and cognitive function. In animal models synaptic plasticity and cognition were improved using the flavanols fisetin (Maher et al., 2006) and (-)epicatechin (van Praag et al., 2007). The ancient decoction Xiaobuxin-Tang has been used as treatment for depressive disorders for centuries and is packed with flavonoids. It increases, supposedly via BDNF, AHN in chronically stressed rats (Adams et al., 2008). In another animal study, a blueberry-rich diet improved spatial working memory via CREB and BDNF activation (Williams et al., 2008). Most interestingly the flavonols rutin and quercetin showed an antidepressant effect in a rat model (Dimpfel, 2009) as well as cocoa polyphenolic extract (Bisson et al., 2008).

The effects of flavonoids have been extensively studied and discussed in several reviews (Spencer, 2010a, b), but will not be discussed in depths here as the focus of this thesis is on the stilbenoid and non-flavonoid Resveratrol.

1.3.2.1 The stilbenoid Resveratrol

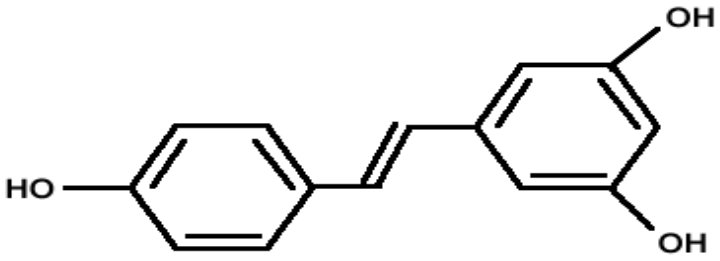


Figure 1-10 Resveratrol (RSVL) 3,5,4'-trihydroxy-trans-stilbenoid

Within the subclass of stilbenes, 3,5,4′-hydroxystilbenoid, or Resveratrol is very well known. The non-flavonoid Resveratrol is a specific polyphenol abundant in the skin of red grapes and red wines and in lower concentrations in peanuts, pistachios, some berries and cocoa. The most dominant source of RSVL is wine

covering 98.4% of the average intake (Zamora-Ros et al., 2008). RSVL is produced by plants as a phytoalexin to protect them from fungal and bacterial invasion. The trans-isomeric form of Resveratrol (Figure 1-10) is by far more commonly found in plants and has been more extensively studied then the cis-isomeric form. It has been suggested to have specific biological properties, including antioxidant, chemopreventive, anti-thrombogenic, anti-inflammatory, cardioprotective, neuroprotective, antiaging and cancer preventive activities (Chachay et al., 2011).

Ghanim et al. showed in two human trails that RSVL (40mg) reduces plasma biomarkers associated with inflammation, can reduce the oxidative and inflammatory response caused by a diet high in fat and carbohydrates and has the potential to reduce the risk of diseases associated with these mechanisms such as diabetes (Ghanim et al., 2010; Ghanim et al., 2011). RSVL improves insulin sensitivity and reduces oxidative stress in diabetic patients (Brasnyo et al., 2011). Another human study found that RSVL increases cerebral blood flow (Kennedy et al., 2010). RSVL has been mostly investigated in several *in vivo* and *in vitro* models. The anti-proliferative, pro-apoptotic effects of RSVL on cancer have been reviewed in (Aggarwal et al., 2004). Most of the animal experiments have been conducted in rat or mouse and the dose range used was from 10mg-200mg/kg body weight. Trauma or ischemic lesion was induced using chemicals or Middle cerebral artery occlusion (MCAO) where an acute dose of RSVL could reduce the lesion area and alleviate associated symptoms (Wang et al., 2004; Appleton et al., 2006; Baur et al., 2006; Airan et al., 2007; Klempin and Kempermann, 2007; Sonmez et al., 2007; Tsai et al., 2007; Adams et al., 2008; Kumar and Sharma, 2010; Singleton et al., 2010; Li et al., 2012). Further, RSVL has been shown to enhance learning and memory (Oomen et

al., 2009; Ranney and Petro, 2009). Recently is has been shown to reverse cognitive behavioural deficits in aged rats. Moreover, working memory correlated with RSVL levels in the hippocampus (Joseph et al., 2008).

RSVL has also been shown to enhance hippocampal neurogenesis (Harada et al., 2011; Moriya et al., 2011). A growing body of *in vivo* evidence indicates that Resveratrol has protective effects in rodent models of stress (Baur and Sinclair, 2006). RSVL interacts with a great number of kinases, receptors and enzymes and pathways which could be responsible for exerting its biological effects. Sirt1 (sirtuin1 or silent mating type information regulation 2 homolog) 1), 5' adenosine monophosphate-activated protein kinase (AMPK), cyclooxygenases and the peroxisome proliferator-activated receptor *(PPAR)* PPARγ have been mentioned times. See also Table 1-2.

In a very compelling rodent study it has been shown that RSVL and the phosphodiesterase type 4 (PDE4)-inhibitor, rolipram -that enhances the effects of the antidepressant sertraline (Anacker et al., 2011)- have similar effects. Rolipram and RSVL treatment, both lead to the same gene expression pattern, improved mitochondrial function, increased physical endurance and basal metabolism and protection against diet-induced obesity and glucose intolerance. They both activate 5' adenosine monophosphate-activated protein kinase (AMPK), Acetyl-CoA carboxylase (ACC) and increase Nicotine adenine dinucleotide (NAD+) and **P**eroxisome proliferator-activated receptor **g**amma **c**oactivator 1-alpha (PGC-1α) levels, suggesting that they increase Sirt1 activity indirectly via the upstream target PDE4 (Park et al., 2012). Sirt1 is an enzyme that deacetylates proteins that contribute to cellular regulation (reaction to stressors, longevity) (Sinclair and Guarente, 2006) and increases lifespan

calorie restricted mice (Haigis and Guarente, 2006) and Saccharomyces cerevisiae (Howitz et al., 2003). See Figure 1-11 for an overview on how RSVL and CR might act on Sirtuins and longevity

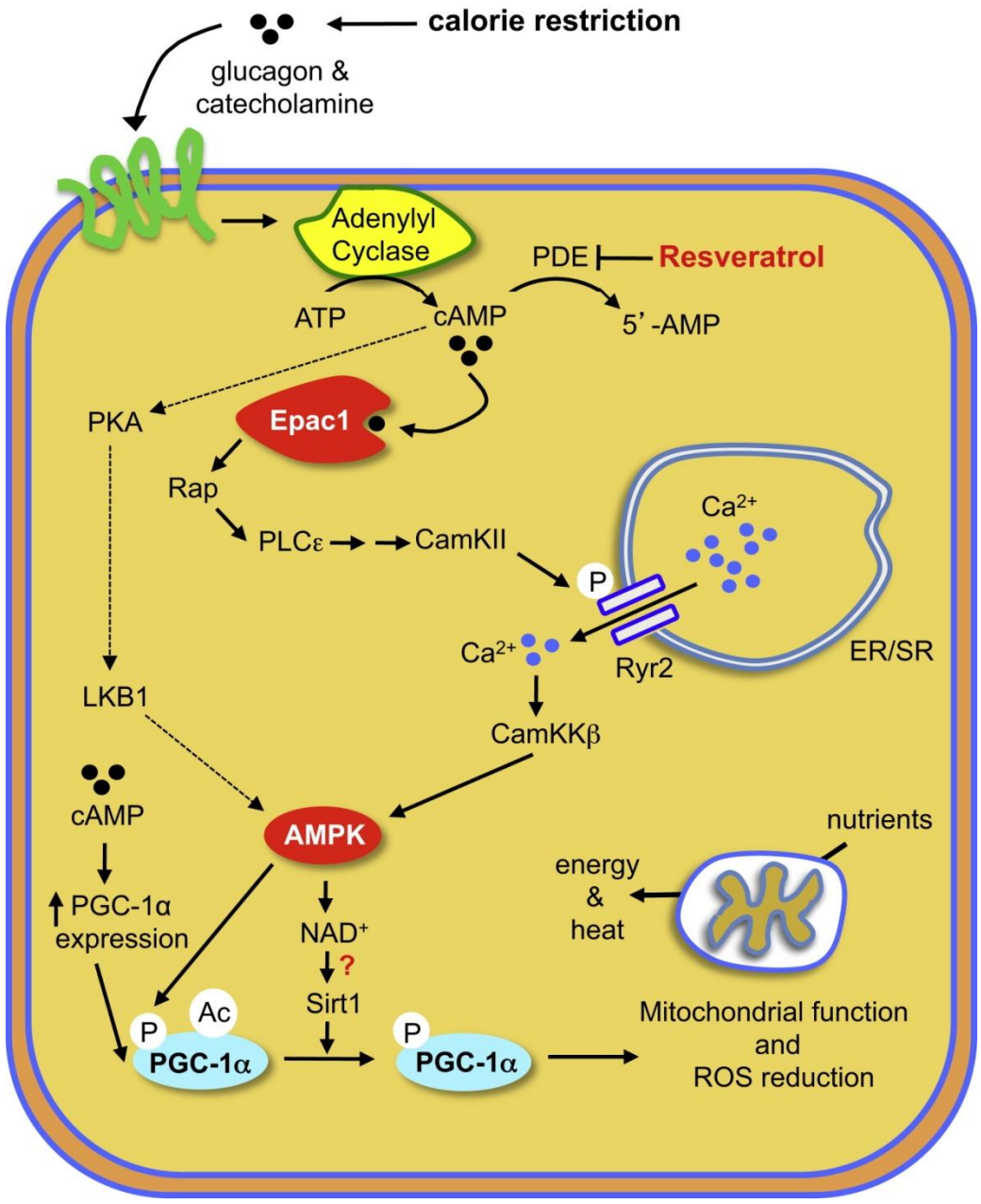


Figure 1-11 Proposed model of how RSVL mimics CR
Resveratrol inhibits PDE activity and induces cAMP signaling and, ultimately, the activation of the CamKKb-AMPK pathway. CR increases cAMP levels by increasing glucagon and catecholamine levels, which activate cAMP production. AMPK increases mitochondrial biogenesis and function by increasing PGC-1a expression, NAD^+ levels, and Sirt1 activity (Park et al., 2012).

Resveratrol also has been shown to activate the transcription factor PPARγ (Peroxisome proliferator-activated receptor) and to protect brain tissue against ischemia (Inoue et al., 2003). In rodents, Resveratrol was shown to significantly ameliorate fatty livers and life spans of obese, diabetic mice (Baur et al., 2006). RSVL improves some symptoms of age related diseases but does not enhance longevity in healthy rodents (Labbe et al., 2011; Miller et al., 2011). In lower organism such as Saccharomyces cerevisiae (yeast), Caenorhabditis elegans (worms), Drosophila melanogaster (fruit flies), RSVL mimics caloric restriction and activates sirtuins and extends lifespan (Wood et al., 2004)

1.3.2.2 Resveratrol as mimetic of caloric restriction

Additionally, it has been shown that Resveratrol delays age-related deterioration and mimics transcriptional aspects of caloric restriction and intermittent fasting (Pearson et al., 2008). Caloric restriction is generally defined as a decrease of 10%-40% of caloric intake compared to ad libitum feeding without malnutrition. Intermittent fasting is also known as alternate day fasting and can be described as alternating eating ad libitum and fasting every other day. Dietary restriction and RSVL have been said to affect lifespan and age related metabolic disorders such as cardiovascular disease, diabetes, obesity and even cancer (Dolinsky and Dyck, 2011; Mercken et al.). Furthermore they have been shown to impact on brain health (Arunachalam et al., 2010; Brownstein et al., 2010; Qiu et al., 2010). Their similar impact on health suggests that they have common molecular targets and pathways. Table 1-2 shows some of the molecular targets that are affected by both caloric restriction and Resveratrol treatment.

molecular signal	Intermittent fasting	calorie restriction	Resveratrol treatment
increased adiponectin		(Shinmura et al., 2007; Kondo et al., 2009; Dolinsky et al., 2010)	(Rivera et al., 2009; Rius et al., 2010)
increased AMPK		(Shinmura et al., 2007; Kondo et al., 2009; Dolinsky et al., 2010; Edwards et al., 2010)	(Baur et al., 2006; Goh et al., 2007; Adams et al., 2008; Dolinsky et al., 2009; Qiu et al., 2010)
increased Akt		(Giani et al., 2008; Sung et al., 2011)	(Hashimoto et al., 2001; Thirunavukkarasu et al., 2007; Tatlidede et al., 2009)
increased endothelial nitric oxide synthase		(Mattagajasingh et al., 2007; Kondo et al., 2009; Dolinsky et al., 2010; Rippe et al., 2010)	(Leikert et al., 2002; Wallerath et al., 2002; Miatello et al., 2005; Nicholson et al., 2008; Penumathsa et al., 2008; Arunachalam et al., 2010; Toklu et al., 2010)
increased Mn superoxide dismutase		(Chandrasekar et al., 2001; Rippe et al., 2010)	(Thirunavukkarasu et al., 2007; Csiszar et al., 2009; Danz et al., 2009; Spanier et al., 2009; Tanno et al., 2010)
reduced IL-6		(Nicklas et al., 2004; Seymour et al., 2006)	(Wang et al., 2001; Inanaga et al., 2009)
reduced NF-κB	(Castello et al., 2010)	(Chandrasekar et al., 2001; Csiszar et al., 2009)	(Rius et al., 2010)
reduced TNF-α		(Nicklas et al., 2004; Seymour et al., 2006)	(Rivera et al., 2009)
increased PGC-1α		(Corton and Brown-Borg, 2005; Anderson et al., 2008)	(Baur et al., 2006; Lagouge et al., 2006; Anderson et al., 2008)
increased SIRT1		(Cohen et al., 2004; Shinmura et al., 2008; Rippe et al., 2010)	(Danz et al., 2009; Kao et al., 2010)
PPARγ activation			(Inoue et al., 2003)

Table 1-2 Common molecular targets of calorie restriction and Resveratrol
Adapted from (Dolinsky and Dyck, 2011)

1.3.3 Dietary restriction

The mechanisms underlying dietary restriction (DR) on lifespan are still unknown, although several pathways have been hypothesised to be involved including: inflammatory processes, oxidative stress, mitochondrial function, apoptosis and body fat composition. Positive effects on longevity have been reported in short lived species such as Saccharomyces cerevisiae (yeast), Caenorhabditis elegans (worms), Drosophila melanogaster (fruit flies), mice, and rats, reviewed in (Colman and Anderson, 2011). However long-term studies in human or non-human primates are needed to elucidate and define the impact of dietary restriction and one long-term study is currently ongoing in human (Calerie http://calerie.dcri.duke.edu/index.html). While preliminary data regarding lifespan from an ongoing non-human primate study are still inconclusive (Colman and Anderson, 2011), many of the general beneficial effects that have been observed in rodents are similar: reduced abdominal adiposity (Colman et al., 2009), improved insulin sensitivity and lipid/lipoprotein profiles (Kemnitz et al., 1994; Lane et al., 1999), decreased oxidative stress (Sohal and Weindruch, 1996). Dietary restriction has been found to exert positive effects also in the CNS (Duan et al., 2001). It has been shown that DR can extend lifespan and improve behavioural outcome in some experimental animal models of neurodegenerative disorders (Mattson et al., 2001). The neuroprotective properties of DR can attenuate brain injury and delay the onset of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Duan and Mattson, 1999; Love, 2005; Patel et al., 2005; Pasinetti et al., 2007).

Also the age-related cognitive decline and molecular mechanisms associated with learning and memory are improved upon dietary restriction (Eckles-Smith

et al., 2000; Fontan-Lozano et al., 2007; Zhao et al., 2007; Witte et al., 2009). Further, cognitive function in rodents is enhanced upon caloric restricted diet (Ingram et al., 1987; Stewart et al., 1989; Gorjao et al., 2007). DR can reverse age and disease-related behavioural and mental impairment (Means et al., 1993; Halagappa et al., 2007; Adams et al., 2008). In rodents it has been observed that AHN increases upon DR (Barnett et al., 2000; Lee et al., 2002) One factor that supports AHN is BDNF which is increased upon dietary restriction in the brain especially in the hippocampus and the cortex (Lee et al., 2000). SIRT1 levels are also elevated upon DR as well as after RSVL treatment (Table 1-2). Another factor that is increased by both RSVL and DR is adiponectin, a endocrine factor that has antidiabetic and insulin sensitising properties (Berg et al., 2002). The effect of dietary restriction on the brain health has been reviewed in (Qiu et al., 2010).

1.3.4 Intermittent fasting (Pilot work)

Although it was assumed that the increased AHN in DR was due solely to reduced calorie intake, unpublished pilot work by Sandrine Thuret's lab has now shown that increased AHN in DR paradigms is not only dependant of calorie intake but also depends on meal frequency in the form of intermittent fasting. Intermittent fasting (IF) is defined as eating every other day ad libitum. Animals were divided into three groups: Ad libitum (AL), Caloric restriction (CR) and Intermittent fasting (IF).

1.3.4.1 Behavioural experiments

The probe trials in the Morris Water Maze for this experiment were 24h and 48h after the acquisition trails. The animals in the three different groups all learn the task and displayed no significant difference in the latency to reach the platform. During the probe trials after 24h all three groups spent significantly more time in the quadrant previously containing the platform; however after 48h only the IF group spent significantly more time in the correct quadrant. This suggests that IF improves retention memory.

1.3.4.2 Cell proliferation and Cell fate

The absolute number of BrdU positive cells per hippocampus is significantly increased after three months of an IF or CR diet compared to an AL diet. Proliferation is increased and the majority of the newly born cells have survived at least 4 week and are now likely to be integrating into the circuit.

Furthermore, the absolute number of newborn neurons is only significantly increased in the F group compared to the AL group. This demonstrates that in

the hippocampus of the IF animals, proliferation and neurogenesis are increased.

1.3.4.3 Gene expression

Microarray and Q-PCR validationshowed that the expression of over 20 genes has been significantly either up or down regulated. The expression of one particular gene: the longevity gene *Klotho* is twofold increased in the hippocampus of mice kept on an IF feeding regime for three months compared to AL or CR.

The molecular effects of dietary modulation on AHN are mediated by specific molecular mechanisms that are still unknown. Therefore the gene expression array data from the Thuret Lab showing changes in gene expression levels upon intermittent fasting in the hippocampus is an excellent starting point to uncover some of these molecular mechanisms. The gene *Klotho* is of particular interest as its mRNA is specifically expressed in the two neurogenic areas, the hippocampus and the SVZ (Figure 1-12), and its expression is up-regulated by twofold upon increased AHN mediated by IF.

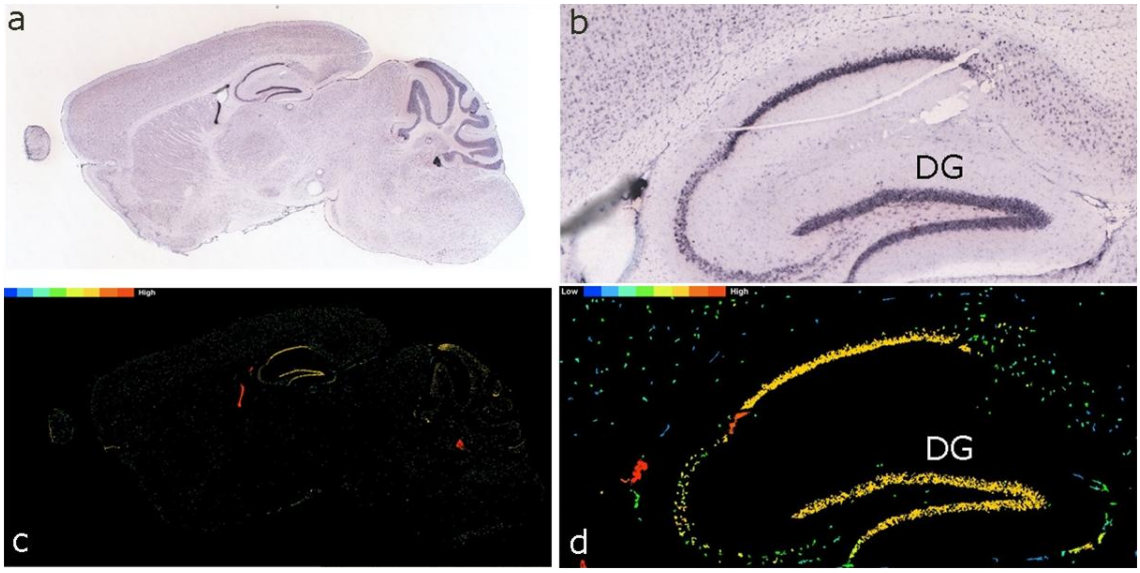


Figure 1-12 Klotho expression in the adult mouse brain
Images show sagital section of adult mouse brain a.) and b.) in situ hybridisation c.) and d.) expression intensity. Available from the Allen brain atlas: http://mouse.brain-map.org.

Klotho is known to be involved in pathways associated with extended live span (Kurosu et al., 2005; Fischer et al., 2010), phosphate excretion involving FGF23, synthesis of Vitamin D in the kidney (Urakawa et al., 2006; Goetz et al., 2010), suppression of growth factor signalling, suppression of oxidative stress, regulation of ion channels and transporters in the kidney (Ikushima et al., 2006; Bloch et al., 2009; Hsieh et al., 2010; Cheng et al., 2011). Most of the published work to date has been conducted in the kidney; however as shown in Figure 1-12 *Klotho* mRNA is very specifically expressed in the hippocampus. All together, these preliminary data and prior published work suggest *Klotho* as an interesting candidate to pursue as a potential molecule mediating the effect of IF, not only on longevity, but particularly on AHN in the context of this thesis.

1.4 Klotho

Klotho is known as the ‘ageing suppressor gene’, due to the symptoms of a mutant mouse resembling human aging (Kuro-o M et al., 1997). In turn in a mouse model over expressing *Klotho* live span is extended up to 30% (Kurosu et al., 2005). Klotho is a single-pass transmembrane protein that is predominately expressed in the kidney (Fischer et al., 2010). The gene *Klotho* shown in Figure 1-13 is composed of 5 exons. The protein consists of two external β-glucosidase-like domains (hKL1 and hKL2), a transmembrane domain and very short cytoplasmic domain. Glucosidases, also called glycosid hydrolases, are enzymes that hydrolyse glycosidic linkages to release small sugars from carbohydrates, glycoprotein and glycolipids. However, it lacks the essential active site Glu residues at positions 241 and 874, suggesting it may be inactive as a glycosidase *in vivo*. The external domain can be shed by alternative splicing of the mRNA and proteolytic cleavage of the protein in the area of Exon 3 leading to a secreted form of Klotho (Airan et al., 2007; Bloch et al., 2009; David et al., 2009). The secreted Klotho has so far been found in blood, urine and Cerebrospinal fluid (CSF) (Imura et al., 2004). For a review see (Wang and Sun, 2009). Table 1-3 shows differences and similarities in genetic location and size in human, mouse and rat. To date the majority of research on Klotho function and expression has been conducted in relation to kidney or cardiovascular diseases in rodents. Recently, German et al. has described the expression of Klotho transmembrane protein in the mouse brain. Klotho is predominantly expressed in the choroid plexus off the SVZ, and cerebellar Purkinje cells. It was found in the plasma membrane as well as near the nuclear membrane (German et al., 2012).

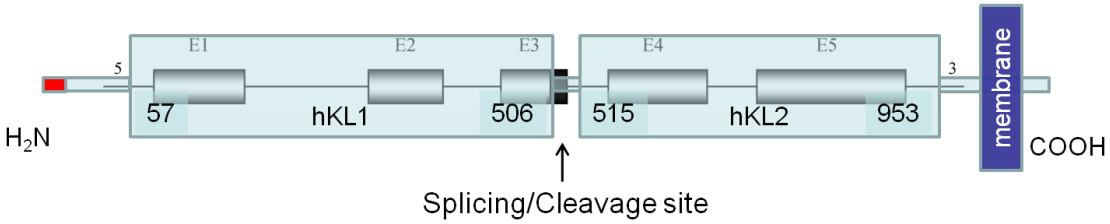


Figure 1-13 Klotho: genomic and protein structure

The gene *Klotho* is composed of 5 exons, resulting in a single-pass transmembrane protein with two β-glucosidase-like domains (hKL1 and hKL2), signal sequence (red), transmembrane domain (blue) and short cytoplasmic domain. A secreted form is genereated by alternative splicing of the mRNA and proteolytic cleavage of the protein in the area of Exon 3

	Human NM_004795	Mouse NM_013823	Rat NM_031336
Gene location	13q12	5 G3	12q12
Membrane form (mRNA) Secreted form (mRNA)	3036 bp 1647 bp	3042 bp 1650 bp	3525 bp
Membrane protein length Secreted protein length	1012aa 549aa	1014aa 550aa	1014aa

Table 1-3: Comparison between human, mouse and rat Klotho gene and protein

The membrane Klotho is necessary for FGF23 signalling for facilitating the high affinity binding of FGF23 to the FGF receptor (FGFR) (Urakawa et al., 2006; Goetz et al., 2010; Cheng et al., 2011). It is therefore not surprising that FGF23-deficient mice and *Klotho* deficient mice display similar phenotypes. FGF23 and *Klotho* have implications in the endocrine regulation of phosphate homeostasis as part of a bone-kidney-parathyroid axis also involving Vitamin D or Calcitriol (Razzaque et al., 2006). This mechanism has been shown to be a key player in chronic kidney disease and ageing (reviewed in (Kuro-o, 2006).

The secreted domain of Klotho acts as a humoral regulator and has pleiotropic functions on glycoproteins located on cell surfaces. It also activates the ion channel Transient Receptor Potential Vanilloid type isoform-5 (TRPV5), which is located in the kidney and has important functions in transephitelial calcium absorption (Aoki et al., 2005; Mensenkamp et al., 2006; Adams et al., 2008). It further activates the Renal Outer Medullary Potassium channel $ROMK_1$ which causes an increase in potassium efflux (Cha et al., 2009). The type-III sodium-dependent phosphate co-transporters Pit-1 and Pit-2 are suppressed by secreted Klotho. They are expressed ubiquitously and mediate cellular phosphate uptake (Hu et al., 2011). Secreted Klotho also regulates several growth factors such as insulin/insulin-like growth factor-1 (IGF1) in L6 myoblast cells or rat hepatoma cells (H4IIE) (Kurosu et al., 2005), Wnt signalling in HEK-293 cells (Liu et al., 2007) and transforming growth factor-β1 (TGF-β1) in mice kidneys and kidney (NRK52E) and lung (A549) cells culture (Doi et al., 2011). Although the underlying molecular mechanisms are not yet fully understood, Insulin/IGF-1 signalling is suppressed by Klotho in the blood of *Klotho* over expressing mice which may be responsible for the anti-ageing properties and

the extended life-span in mice over expressing *Klotho* (Ballen et al., 2000; Kenyon, 2005; Kurosu et al., 2005; Kirstetter et al., 2006; Scheller et al., 2006; Chateau et al., 2010). However, data from the Vischer lab suggested that decreased *Klotho* expression is not a general feature of rodent models of insulin resistance and that the soluble Klotho protein does not inhibit IGF-1 and/or insulin signalling in HEK293, L6, and HepG2 cultured cells, arguing against a direct role of Klotho in insulin signalling (Lorenzi et al., 2010). The mechanism by which Klotho impacts on insulin signalling needs yet to be determined. Wnt signalling is inhibited by amino-terminal portion of Klotho's KL1 extracellular domain (amino acids 1 to 285) binding directly to Wnt ligands prohibiting its activation in hematopoietic stem cells (HSC) and HEK-293 cells. (Ballen et al., 2000; Kirstetter et al., 2006; Scheller et al., 2006). Wnt signalling plays a key role during proliferation in the adult hippocampus (Lie et al., 2005). In *Klotho* mutant mice Wnt signalling is amplified which leads to an exhaustion of the stem cell pool in primary mouse embryonic fibroblasts and HSC.

Interestingly, *Klotho* has been shown to be a target gene of PPARγ, a transcription factor activated by Resveratrol (Zhang et al., 2008). Peroxisome proliferator-activated receptors are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes (Greene et al., 1995). PPARs play essential roles during cellular differentiation, development metabolism and tumorigenesis (Fajas et al., 1997). PPARγ is further a target gene of RSVL in a mouse model (Inoue et al., 2003) and is highly expressed in the SVZ, the hippocampus and the RMS (Morales-Garcia et al., 2011). PPARγ and *Klotho* therefore provide a potential link between RSVL and AHN.

Up to now, nothing was known about the impact of Klotho on AHN and its action in the brain. However, as described in section 1.3.4, the Thuret Lab found that *Klotho* expression is increased upon intermittent fasting in the hippocampus in mice leading to an improved retention memory.

Altogether this makes *Klotho* an interesting candidate for studying its role in the regulation of adult hippocampal neurogenesis and depression.

1.5 PPARγ

Peroxisome proliferator-activated receptors are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes (Greene et al., 1995) during cellular differentiation, development metabolism and tumorigenesis (Fajas et al., 1997). PPARs form heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various genes. Three subtypes of PPARs are known: PPARα, PPARδ, and PPARγ. PPARγ is predominantly expressed in adipose tissue, macrophages, colon epithelium and also in small intestine and skeletal muscle, explaining its implication in the pathology of numerous diseases including obesity, diabetes, atherosclerosis and cancer. All three PPARs are expressed at different levels and areas in the rat brain; PPARγ is expressed in the granular cells in the DG (Braissant et al., 1996) and as shown in Section 2.1.1 in HPC03A/07. Activation of PPARγ up-regulates neural stem cell proliferation and differentiation *in vivo* and *in vitro* (Morales-Garcia et al., 2011). *Klotho* has been shown to be a target gene of PPARγ in HEK293 cells (Zhang et al., 2008) and moreover PPARγ is a target gene of RSVL in murine primary cortical cultures, bovine brain microvessel vascular endothelial cells (BBMEC), human umbilical venous endothelial cells (HUVEC) and bovine arterial endothelial cells (BAEC) (Inoue et al., 2003). PPARγ and Klotho therefore provide a potential link between diet (RSVL) and AHN. The question addressed in Section 0 is whether RSVL acts exclusively via a PPARγ and Klotho dependent pathway or whether RSVL affects Klotho and AHN via a PPARγ independent pathway. RSVL has similar effects on longevity and health as CR and IF which suggests that they have common molecular targets and pathways. Table 1-2 shows some of the

molecular targets that are affected by both caloric restriction and Resveratrol treatment. Increasing the RSVL uptake using supplements is more feasible for human beings than living on a by 30-40% CR or IF diet. Further using RSVL might help to discover pathways that will uncover the molecular mechanism of increased AHN and longevity involving PPARγ and Klotho or even new, yet unknown pathways.

1.6 Summary and hypothesis

Based on the evidence above I hypothesis that Omega-3, Omega-6 FA, Resveratrol, intermittent fasting, PPARγ and Klotho are involved in regulating AHN and ultimately might be able to influence depressive behaviour. Uncovering the cellular mechanisms by which Omega-3, Omega-6 FA, RSVL and Klotho modulate AHN could provide new fundamental molecular and cellular data on the functioning of depression and eventually help to prevent and support its treatment. Figure 1-14 gives a brief overview of how RSVL, Klotho and PPARγ act on AHN to the knowledge before this thesis.

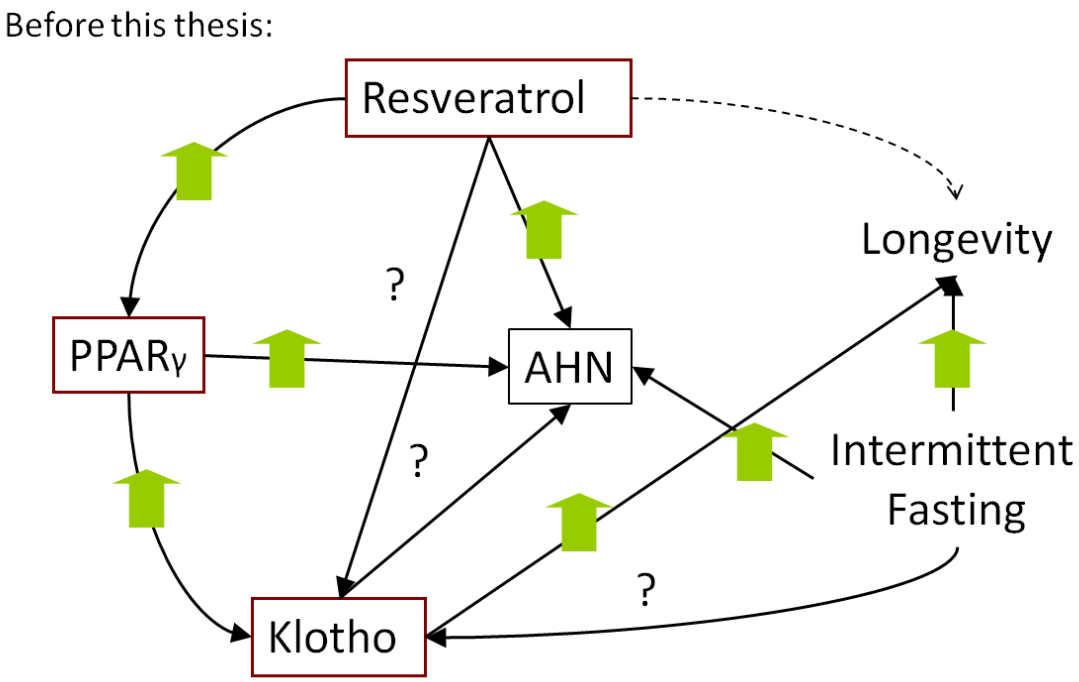


Figure 1-14 Working hypothesis: Linking AHN, Klotho, PPARγ, Resveratrol and intermittent fasting
Green arrows: upregulation or activation, Question marks: not known yet

1.7 Aims

1.7.1 Aim 1

Diet in form of Omega-3 fatty acids is known to have beneficial effects on learning/memory and mood. Interestingly, it has been hypothesised that anti-depressants may exert their effects by increasing AHN. This raised the possibility that certain diets may help protect against depression via modulating AHN. To determine the possible preventative properties of Omega-3s and RSVL, an *in vitro* model of stress was employed where cells were treated with cortisol to induce stress and decrease proliferation and neurogenesis in HPCOA07/03 (Section 2.1.5.1). The ability of these compounds to overcome the effect of Cortisol will provide evidence that diet can potentially be beneficial for depression via modulating neurogenesis.

1.7.2 Aim 2

The expression of *Klotho*, the longevity gene, is increased upon intermittent fasting (IF) (section 1.3.4) and correlates with increased AHN and retention memory (section 1.3.4). To determine whether *Klotho* has a direct influence on AHN, this gene was over-expressed or suppressed in HPCOA07/03 cells *in vitro*. These experiments will provide insight in the role of *Klotho* on proliferation and differentiation.

1.7.3 Aim 3

To further investigate the molecular pathways regulating *Klotho* expression I focused on PPARγ, a nuclear receptor transcription factor known to activate *Klotho*. Interestingly, Resveratrol is known to activate PPARγ. After I have shown under Aim 1 that RSVL increases proliferation and differentiation in

HPCOA07/03 cells, in these experiments the ability of RSVL to activate *Klotho* via PPARγ was investigated in HPCOA07/03 where either *Klotho* or PPARγ expression was knocked down or blocked respectively. These experiments will test the hypothesis that PPARγ provides a direct link from diet (RSVL) to AHN.

Chapter 2 Materials and Methods

If not stated in the text, please see Appendix for a detailed list of all the material, reagents and the concentrations used in this thesis.

2.1 Tissue culture

2.1.1 The embryonic hippocampal progenitor cell line HPC03A/07

To conduct the experiments in this thesis, the human fetal hippocampal progenitor cell line HPC03A/07 from ReNeuron (Surrey, U.K.) was used. This neural progenitor cell line (NPC) was used as an *in vitro* model of hippocampal neurogenesis to assess the effects of Omega-3 fatty acids, Omega-6 fatty acids, RSVL and the gene *Klotho* on cell proliferation and fate, and to begin to elucidate the molecular mechanisms governing their mode of action. Cells were cultured at 37˚C, in saturated humidity and 5% CO_2 and medium was exchanged fully every second day.

The human foetal hippocampal progenitor cell line HPC03A/07, is a karyotypically normal human NPC line generated from cells isolated from a first trimester foetal brain. It is a conditionally immortalised multipotent progenitor cell line with the ability to self-renew for long periods of time and to differentiate into specialised cells with specific functions. However, these cells are limited in their ability to differentiate, meaning that they can give rise to neural cells, glia and very few oligodendrocytes, but not to other cell types outside of the brain. HPC03A/07 cells contain the c-myc-ER^{TAM} sequence that is conditionally activated by 4-hydroxytamoxifen (4-OHT) and controls cell proliferation and differentiation. See Figure 2-1. Grown in the presence of 4-OHT and the growth factors recombinant human Fibroblast Growth Factor-basic (FGF2) and recombinant human epidermal Growth Factor (EGF) the cells express the gene c-myc within the nucleus. Under these conditions the cells proliferate indefinitely

and produce stable cell lines in culture. Once the growth factors and the 4-OHT are removed from the media the cells cease to proliferate and start to differentiate (Pollock et al., 2006).

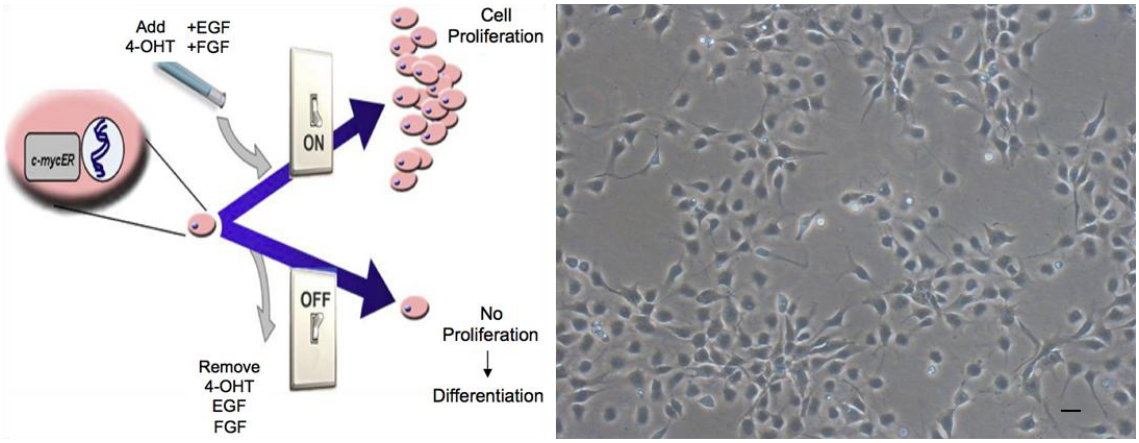


Figure 2-1 ReNeuron's HPC03A/07 cells with c-mycER technology.
The human foetal hippocampal progenitor cells stably express the c-mycER transgene and proliferate in the presence of the growth factors EGF and bFGF, and the synthetic compound 4-hydroxytamoxifen (4-OHT) which activates c-mycER. Upon removal of EGF, bFGF and 4-OHT, HPC03A/07 cells stop to proliferate and start to differentiate into neurons, astrocytes and oligodendrocytes. (Scalebar: 20µm) Picture source: www.reneuron.com

The HPC03A/07 cells were cultured as previously described by (Johansson et al., 2008) with minor modifications, in DMEM:F12 medium supplemented with a range of components here referred to as full growth medium (Table 7-1). Cells were grown on laminin (Sigma 20µg/ml) coated nunc flasks, nunc 96 well plates (both Nunclon, Roskilde, Denmark) or glass slides with reusable chambers (flexiPERM, Greiner bio-one) at a seeding density of $1.6x10^4/cm^2$ ($1.2 x10^6$ per nunc T75 flask). In the proliferating state the cells had a doubling time of 72h and were therefore passaged every 72 hours at 70-80% confluence (Johansson et al., 2008). Cells were passaged using 1x Trypsin/EDTA (Invitrogen) for 5min at 37°C followed by soya bean trypsin inhibitor 0.5 mg/ml (Sigma). The suspended cells were centrifuged in a centrifuge tube (Corning) (900g for 5min at room temperature (RT)), resuspended in full growth medium and plated at an appropriate concentration as stated above.

Below the basic timeline for growing the HPC03A/07 cells is described, however this may vary depending on the experiment. Please see the timelines for each set of experiments in the results chapter. For the proliferation assay cells were cultured for 3 days under proliferation conditions. For the differentiation assay medium without growth factors and 4-OHT was used and cells were differentiated for 3 days or 7 days. These times have initially been chosen to capture differentiation at an early and later stage, however after initial experiments only the 7 day differentiation time point was used for further experiments as no significant differences could be seen between 3 days and 7 days. See Figure 2-2 and the experimental timeline for each set of experiments for details. To be able to clearly see changes during early neuronal development caused by *Klotho*, Omega-3/6 fatty acids or RSVL, no neurotrophic factors to artificially promote neuronal differentiation were added.

This model therefore excludes such compounds as confounding factors from the analysis.

To validate the usefulness and appropriateness of the HPCO3A/07 cell line for my studies, I had to confirm the expression of genes of interest (*Klotho*, PPARγ) and protein of interest (*Klotho*) in this cell line as well as establish the immunocytochemical profile during proliferation and differentiation of the HPCO3A/07 cell line.

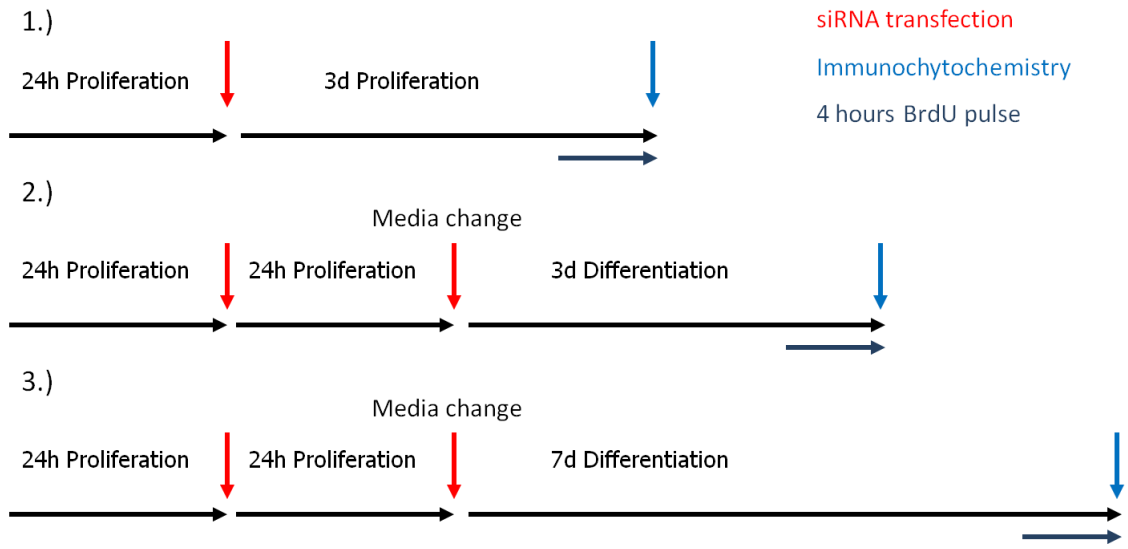


Figure 2-2 Timeline for growing HPC0A07/03A cells.
1.) Proliferation assay, 2.) 3 day Differentiation assay 3.) 7 day Differentiation assay

2.1.2 Immunocytochemical cell culture characteristics of HPC03A/07 during proliferation and differentiation

To assess the phenotypic characteristics of HPC03A/07 during proliferation, cells were allowed to proliferate for three days before they were fixed and stained with a range of indicative markers: the proliferation marker Ki67; the stem cell markers SOX2 and Nestin; the apoptosis marker activated Caspase-3, as well as Klotho, the protein of the longevity gene (my protein of interest; see section 2.2 for details). In all cases, DAPI was used as a nuclear counter stain (Figure 2-3). After 3 days under proliferation conditions condition (Figure 2-3), 50.2±2.9% of the cells expressed Ki67, while 100% of the cells expressed SOX2 and Nestin (Figure 2-3a-d). 3.4±0.5% of the total cells expressed Klotho and 3.3±0.7% express activated Caspase-3 (Figure 2-3e-g). This suggests that 50% of the cells are dividing and all cells express stem cell markers. Klotho and activated Caspase-3 are expressed in a very small proportion of the cultures.

To determine the immunocytochemical characteristics of HPC03A/07 during differentiation, cells were differentiated for seven days and then stained as described above. These differentiated cells were further stained for Dcx (neuroblasts/early neurons marker), MAP2 (mature neurons marker) and the astrocyte marker S100b. Figure 2-4 shows that after 7 days of differentiation the proportion of Ki67 expressing cells decreased to 21.8±2.8%, as might be expected, however the percentage of both SOX2 and Nestin expressing cells remained at almost 100 % (SOX2: 99.4±0.3%, Nestin: 99.1±0.3%,), although differentiation was started. The SOX2 and/or Nestin proteins might not have been degraded after 7 days. A longer differentiation period might be necessary for the NPC marker to degrade. Within the experiments described in the subsequent chapters, their expression never changed except under conditions

where *Klotho* was over expressed in the HPC03A/07cells (see 4.2). Upon differentiation, the proportion of Klotho expressing cells more than doubled to 7.9±1.8%, the percentage of activated Caspase-3 positive cells did not change with 3.2±0.5%. The astrocyte marker S100b was expressed in 13.6±0.7% of the cells. Further, the early neuronal marker Dcx was expressed in 14.4±0.2% and MAP2, a marker for mature neurons in 11.2±0.3% of the total cells (Figure 2-5). These results suggest that SOX2 and Nestin positive cells must co-label with Klotho, astrocytic and neuronal markers. In this thesis cells were only co-labelled for SOX2 and Nestin but not for SOX2 or Nestin with marker for dividing or differentiating cells. However, *in vivo* studies show that neuronal markers, such as Dcx, MAP2, NeuroD1, NeuN and Tuj1 are mutually exclusive with SOX2, however NeuroD1 positive cells do co-localise with Nestin in the hippocampus of adult mice (Suh et al., 2007; Kuwabara et al., 2009). Throughout experiments using the HPC03A/07 cell line conducted by colleagues in the department and elsewhere, the SOX2 and Nestin proportion did not decrease after 7 days of differentiation. As described above, 7 days of differentiation might not be long enough for SOX2 and Nestin to be degraded, leading to fate committed cells still expressing SOX2 and Nestin. Co-labelling with markers for dividing and differentiating cells as well as extending the differentiation period would be interesting experiments to further characterise this cell line. Further, BrdU labelling to determine proliferation rate has been conducted. However due to technical problems for certain replicates, the BrdU experiments will not be presented in this thesis.

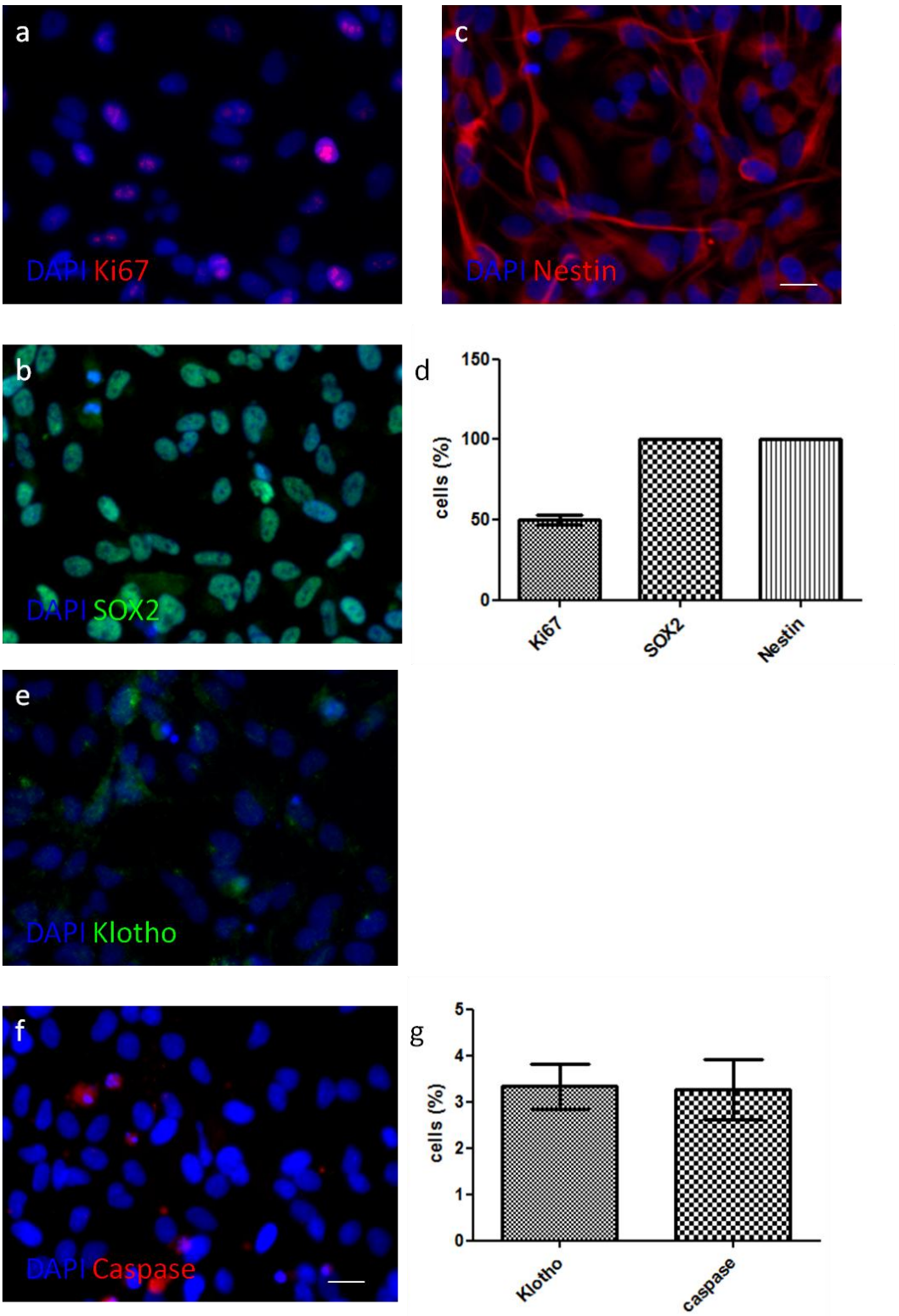


Figure 2-3 Expression of Ki67, SOX2, Nestin, Klotho and activated Caspase-3 in HPC03A/07 cells after 3 days under proliferation conditions.
a.) DAPI and Ki67 b.) DAPI and SOX2 c.) DAPI and Nestin d.) Quantification of the percentage of DAPI cells expressing Ki67, SOX2 and Nestin e.) DAPI and Klotho f.) DAPI and activated Caspase-3 h.) Quantification of the percentage of DAPI cells expressing Klotho, activated Caspase-3. (Scalebar: 20µm, Error bars are the Standard Error of the Mean (SEM).

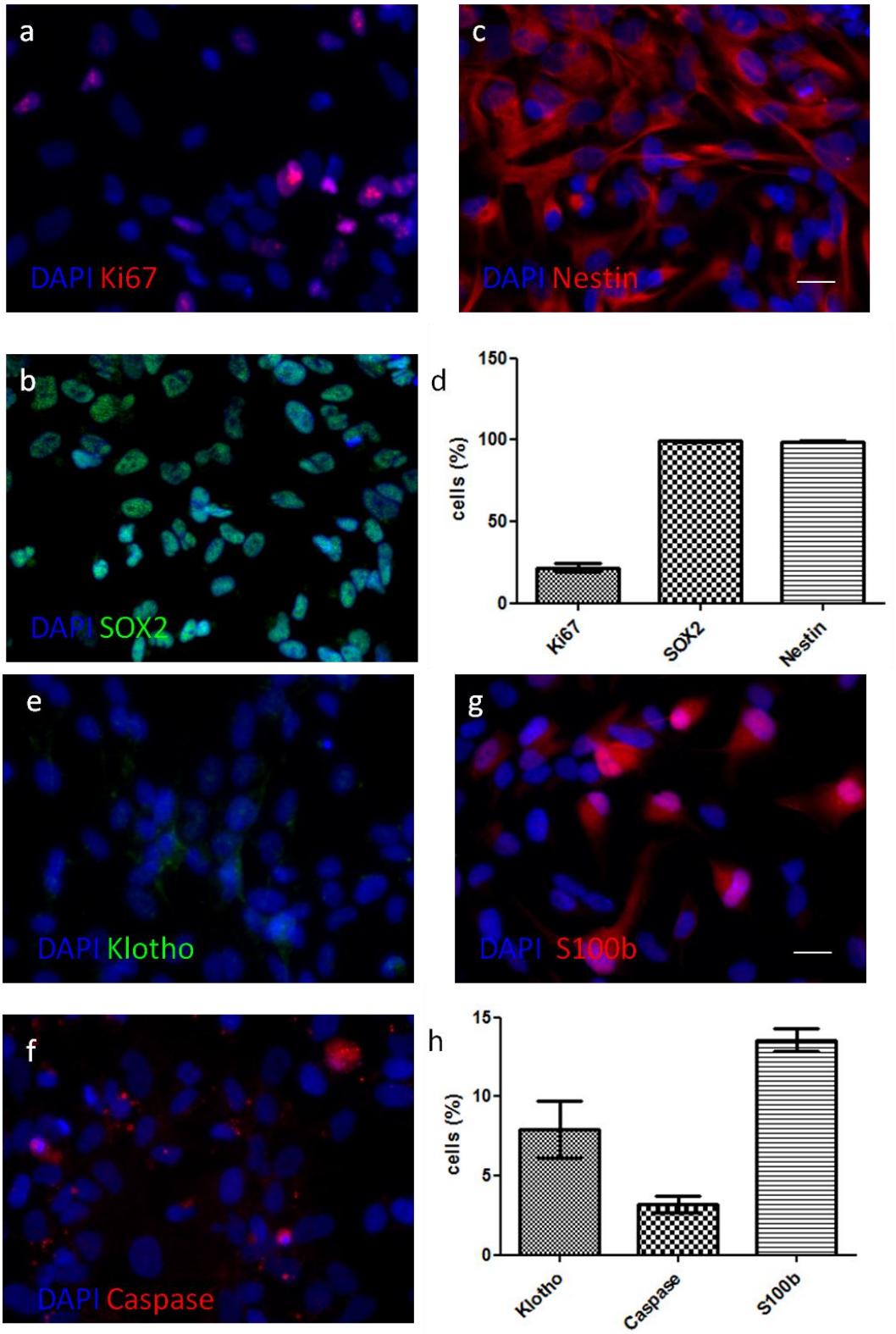


Figure 2-4 Expression of Ki67, SOX2, Nestin, Klotho, activated Caspase-3 and S100b in HPC03A/07 after 7 days under differentiation conditions.
a.) DAPI and Ki67 b.) DAPI and SOX2 c.) DAPI and SOX2 d.) Quantification of the percentage of DAPI cells expressing Ki67, SOX2 and Nestin. e.)DAPI and Klotho f.) DAPI and activated Caspase-3 g.) DAPI and S100b h.) Quantification of the percentage of DAPI cells expressing Klotho, activated Caspase-3 and S100b. (Scalebar: 20µm, Error bars are the Standard Error of the Mean (SEM).

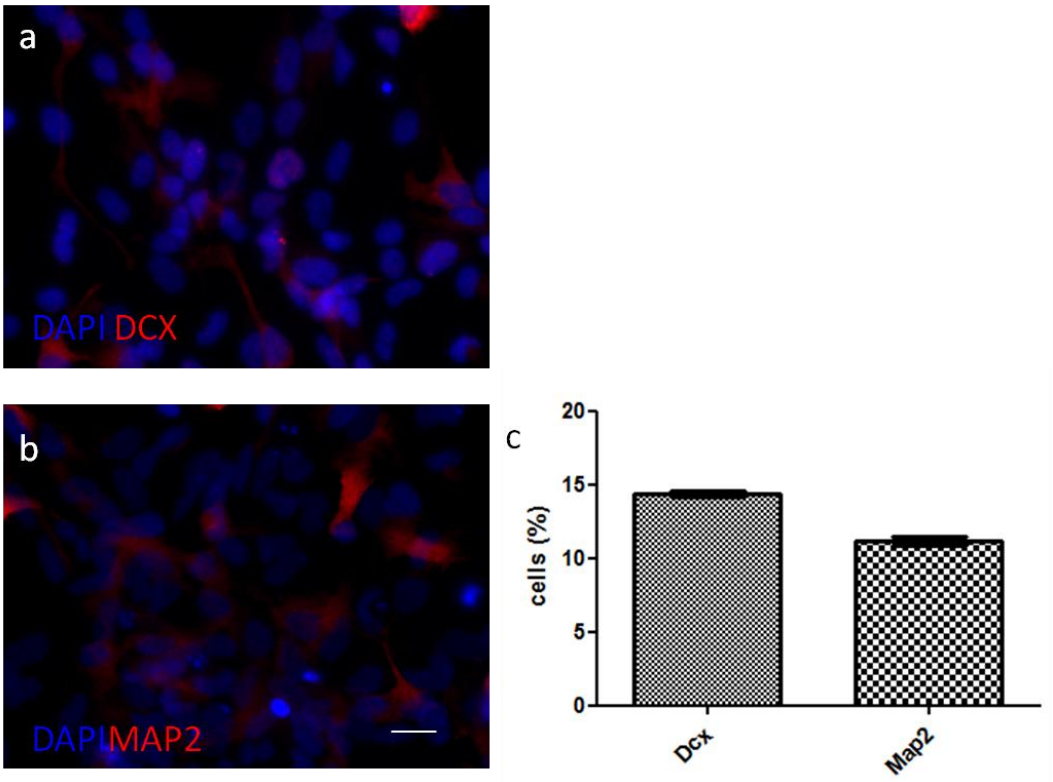


Figure 2-5 Expression of Dcx and MAP2 in HPC03A/07 after 7 days under differentiation conditions.
a.) DAPI and Dcx b.) DAPI and MAP2 c.) Quantification of the percentage of DAPI cells expressing Dcx and MAP2. (Scalebar: 20µm, Error bars are the Standard Error of the Mean (SEM).

2.1.3 HPC03A/07 cells express Klotho and PPARγ mRNA

To determine the influence of *Klotho* on hippocampal neurogenesis its expression was either knocked down or over-expressed. Details of this analysis are presented in Section 0. As only a small percentage of cells express Klotho I wanted to determine the level of *Klotho* mRNA to be able to quantify the level of *Klotho* knock down or over-expression respectively.

HPC03A/07 cells were maintained for 3 days under proliferation conditions or for 3 and 7 days under differentiation conditions before RNA was extracted and processed as described in section 2.4 to generate cDNA. For the PCR SYBR green was used. All details are described under section 2.4.4. *Klotho* mRNA was detected in HPC03A/07 cells under all three conditions. However, its expression was very low and the Ct value was out of the dynamic range, making an accurate quantitative analysis for the knock down experiment very difficult. (Data not shown)

However, despite the small proportion of cells expressing Klotho protein and the low expression of *Klotho* mRNA these HPC03A/07 cells were still a good model to study Klotho as the immunocytochemistry is very clear and when *Klotho* is over expressed immunocytochemistry, mRNA (Figure 4-4) and also Western Blot (Figure 4-5) results are well-defined. Therefore these cells may be not an ideal model for *Klotho* knock down but a very good model for *Klotho* over-expression, which will allow me to assess the effect of Klotho on proliferation and differentiation in HPC03A/07.

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes (Greene et al., 1995). PPARs play essential roles during cellular

differentiation, development, metabolism and tumorigenesis (Fajas et al., 1997). Furthermore, PPARγ is a target gene of RSVL (Inoue et al., 2003). And moreover *Klotho* has been shown to be a target gene of PPARγ (Zhang et al., 2008). PPARγ and Klotho therefore provide a potential link between diet in the form of RSVL and AHN. To be able to investigate the role of PPARγ in relationship to RSVL and Klotho, I wanted to confirm the expression of PPARγ in this model, using Q-PCR (SYBR Green). PPARγ is expressed in HPC03A/07 cells upon 3 day proliferation and 7 day differentiation (Ct 27). (Figure 2-7)

2.1.4 Summary

HPC03A/07 cells express Ki67 and the neural progenitor cell marker SOX2 and Nestin under proliferative conditions. Additionally, they express the early neuronal marker Dcx and the mature neuronal marker MAP2 under differentiation conditions. Also S100b a marker for astrocytes is expressed under differentiation conditions.

Figure 2-6 shows a summary of Klotho (images) and PPARγ expression during proliferation and differentiation. *Klotho* mRNA and protein are expressed in HPC03A/07 after 3 days of proliferation as well as after 3 days and 7 days differentiation. Klotho co-labels with Ki67 (Figure 4-11c), Dcx (Figure 4-9c), MAP2 (Figure 4-9d) and activated Caspase-3 (Figure 4-12c) in some cells but never with S100b (Figure 4-10c). This indicates that Klotho is expressed in proliferating, differentiating and apoptotic cells but not in astrocytes. PPARγ mRNA was expressed after 3 days proliferation and after 3 and 7 days differentiation. See Figure 2-7.

This demonstrates that HPC03A/07 cells make a good system to investigate the effect of Klotho, RSVL and PPARγ on cell proliferation and cell fate. Further it is a good model to study the effects of Omega-3 fatty acids on cell proliferation and fate as it is a human hippocampal cell line that allows extrapolation from the results *in vitro* to changes in the hippocampus and to their possible impact on behaviour.

In vitro	3 days proliferation	3 days differentiation	7 days differentiation
Klotho mRNA	+	+	+
Klotho protein	+	+	+
PPARγ mRNA	+	+	+

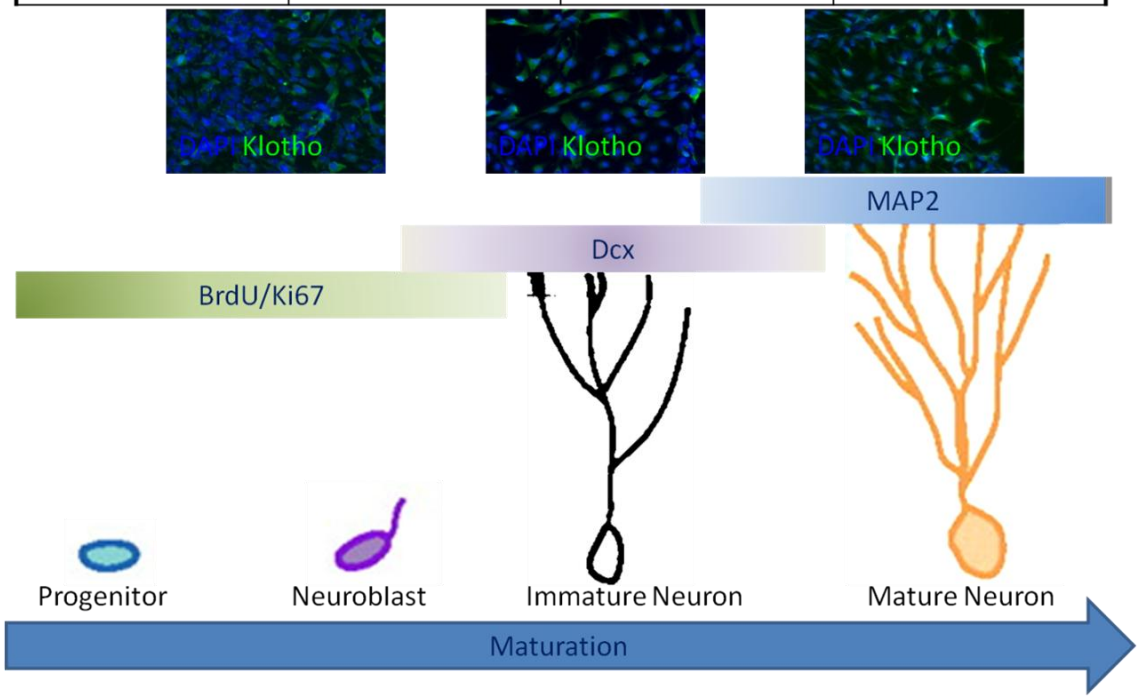


Figure 2-6 Summary of *Klotho* expression in HPC03A/07
Klotho mRNA and protein is expressed in cultures after 3 days proliferation and after 3 days and 7 days differentiation

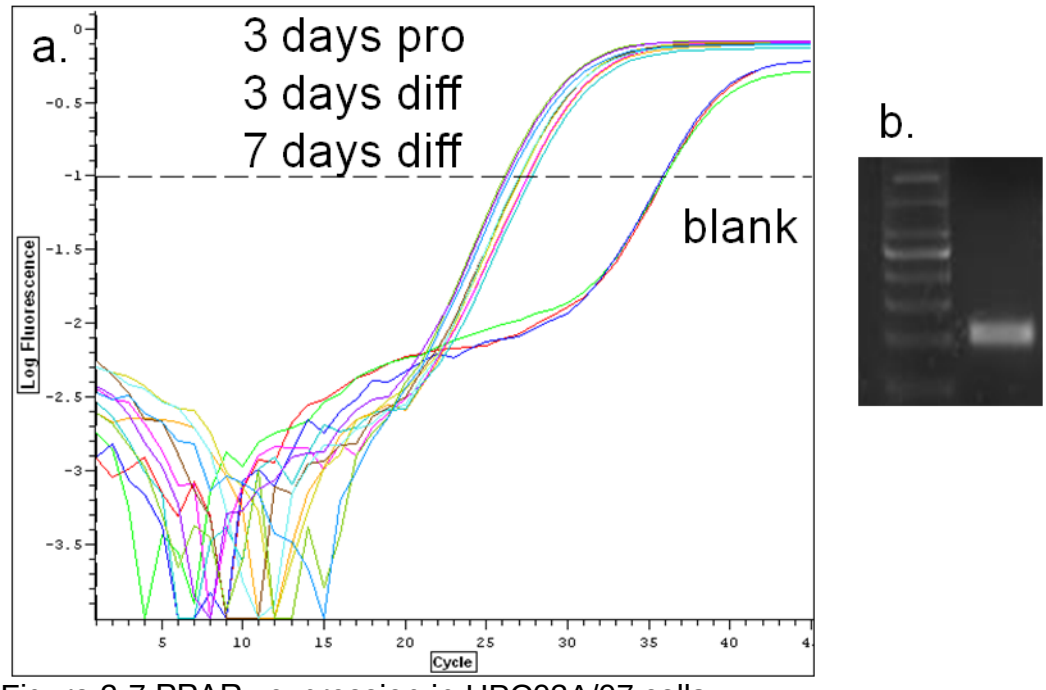


Figure 2-7 PPARγ expression in HPC03A/07 cells
a.) CT values using SYBRgreen b.) band for 3 days proliferation on gel

2.1.5 Resveratrol and Omega 3 and Omega 6 fatty acids treatment

To assess the effect of the stilbenoid Resveratrol and Omega-3s and Omega-6s in Table 2-1 on proliferation and differentiation, cells were seeded in 96 well plates for immunochytochemistry as described before and cultured for 24h in growth medium, before treatment was applied. Fatty acids and Resveratrol (Sigma) were dissolved in 100% Ethanol and diluted in growth medium to give a final concentration as indicated in Table 2-1. The growth medium containes human albumin, which binds the fatty acids and facilitates their transport and delivery into the cells. For the proliferation assay cells were further cultured for 6 days under proliferation conditions. For the differentiation assay differentiation was started 6days after seeding and compounds were added at the same time. Cells were cultured under differentiation conditions for 7 days. These experiments were carried out to screen for the fatty acid with the most effect. After these experiments only EPA, DHA and RSVL were used for further experiments used in this thesis.

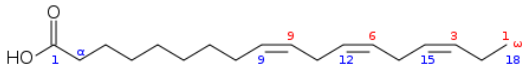
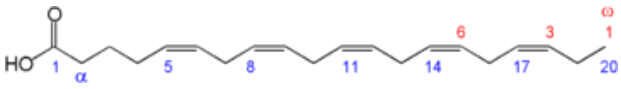
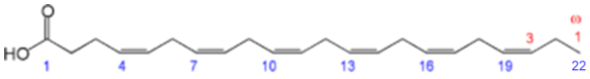
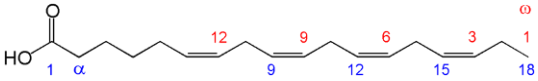
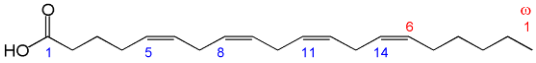
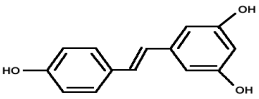
α-Linolenic Acid (ALA) (18:3 n-**3**) 10µM	
Eicosapentaenoic Acid (EPA) (20:5 n-**3**) 10µM	
Docosahexaenoic Acid (DHA) (22:6 n-**3**) 10µM	
Stearidonic Acid (SDA) (18:4 n-**3**) 2µM	
Arachidonic Acid (AA) (20:4 n-**6**) 10µM	
Resveratrol (RSVL) 1µM	

Table 2-1 Omega-3, Omega-6 fatty acids and RSVL

Overview of reagents and their concentrations used in this study. All compounds are solved in 100% ethanol. Medium contains Albumin facilitating transport and delevery into the cell.

2.1.5.1 Cortisol stress model

Glucocorticoid hormones, such as the human endogenous glucocorticoid, Cortisol, are consistently elevated in severely depressed patients and in animal models of chronic stress and depression. Importantly, high levels of glucocorticoid hormones have detrimental effects on learning, memory and mood and decrease adult hippocampal neurogenesis in rodents (Mirescu and Gould, 2006; David et al., 2009; Pariante, 2009). Diet in form of Omega-3 fatty acids is known to have beneficial effects on learning, memory and mood. Interestingly, it has been hypothesised that anti-depressants may exert their effects by increasing AHN. This raised the possibility that Omega-3 fatty acids may help to protect against depression by increasing proliferation and differentiation. Christoph Anacker (SPI lab, IOP, King's College London) showed that proliferation and differentiation in HPC cells was decreased after treating them with high concentration (100μM) of Cortisol (Sigma). See Figure 2-8. He also showed that Cortisol only decreases proliferation and neurogenesis when cells are treated during the mitotic phase, which is why I treated the cultures with Cortisol for 3 days under proliferation conditions before starting differentiation.

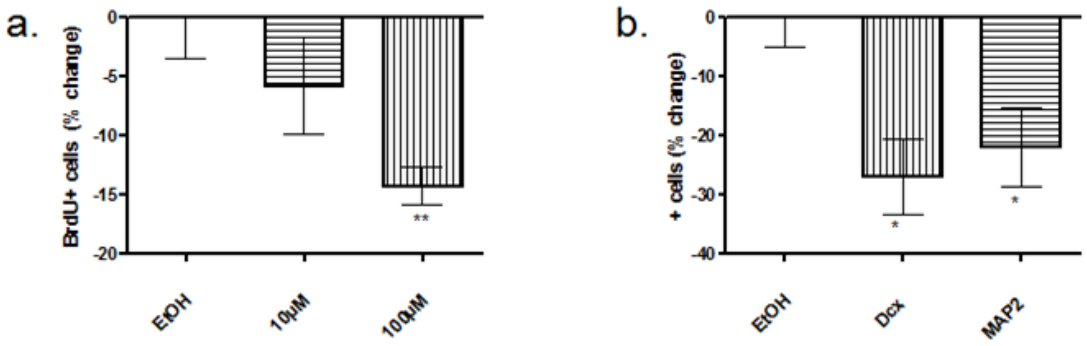


Figure 2-8 Cortisol decreases proliferation and differentiation in HPC03A/07 cells

a.) Proliferation and b.) Differentiation are significantly decreased in HPC03A/07 after treatment with high concentrations of Cortisol (100µM).

Using these results I developed an *in vitro* stress model were high concentrations of Cortisol were used to mimic the increased Cortisol levels seen in depressed patients and in animal models of depressive behaviour.

To determine the possible preventative properties of Omega-3s and RSVL using this *in vitro* stress model HPCOA03/07A were pre-treated with EPA, DHA or RSVL for 3 days under proliferating conditions before treatment with 100µM Cortisol was started. See Figure 2-9. EPA, DHA or RSVL treatment was either continued in parallel to Cortisol treatment for a further 3 days of proliferation or stopped after the pre-treatment of 3 days of proliferation before cultures were fixed for immunocytochemistry (section 2.2). For the differentiation assay EPA, DHA or RSVL treatment was either continued for 6 days of proliferation and 7 days differentiation or stopped after the pre-treatment or after 6 days of proliferation before fixation with 4% PFA. This resulted in different experimental combinations for the proliferation assay as well as the differentiation assay as described in Table 2-2 and Table 2-3. Control cultures were treated with the equivalent concentration of Ethanol (1%).

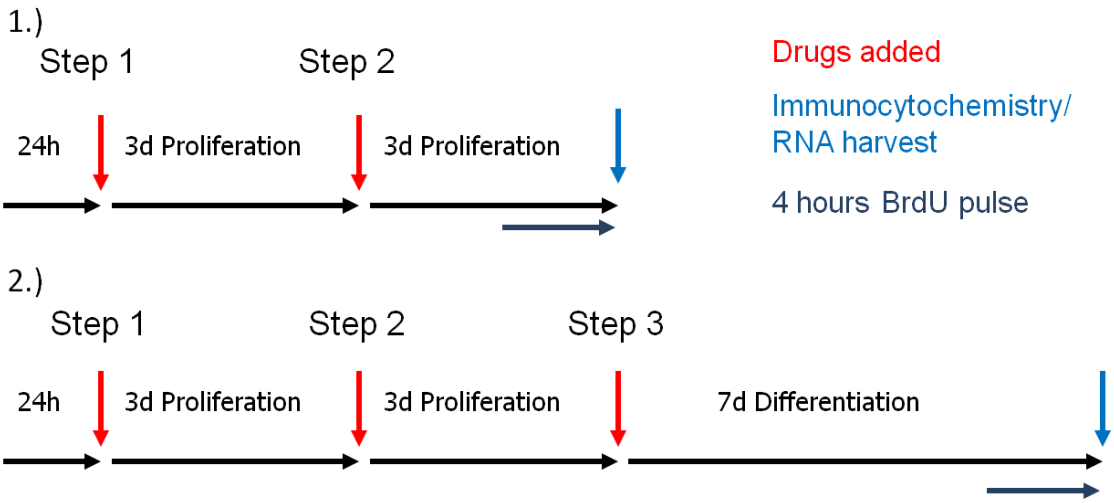


Figure 2-9 Timeline for Omega 3 prevention experiment using the Cortisol stress model on HPC0A07/03A cells.
1.) Proliferation assay, 2.) Differentiation assay Step 1: Cells were pre-treated with EPA, DHA or RSVL for 3 days under proliferation conditions. Step 2: Cortisol was added and EPA, DHA or RSVL treatment was either stopped or continued. For the proliferation assay cells were fixed for immunocytochemistry after an additional 3 days proliferation. Step 3: For the differentiation assay differentiation was started now and EPA, DHA or RSVL treatment was either stopped or continued leading to different combinations as described in Table 2-2 and Table 2-3

1.) Proliferation assay

	Step 1	Step 2
1.	3d pro O3	3d pro O3 Cort
2.	3d pro O3	3d pro Cort
3.	3d pro	3d pro Cort
4.	3d pro O3	3d pro O3
5.	1% EtOH	

Table 2-2 Conditions for the omega-3 prevention experiment using the Cortisol stress model under proliferating conditions.
Step 1: Cells were pre-treated with O3 for 3 days of proliferation, Step 2: Cortisol was added and O3 treatment was, depending on the condition continued or stopped before cultures were fixed. O3: DHA, EPA, RSVL; Cort: Cortisol; Pro: Proliferation

2.) Differentiation assay

	Step 1	Step2	Step 3
1.	3d pro O3	3d pro O3 Cort	7d diff O3 Cort
2.	3d pro O3	3d pro O3 Cort	7d diff Cort
3.	3d pro O3	3d pro Cort	7d diff Cort
4.	3d pro	3d pro Cort	7d diff Cort
5.	3d pro O3	3d pro O3	7d diff O3
6.	1% EtOH		

Table 2-3 Conditions for omega-3 prevention experiment using the Cortisol stress model under differentiation conditions.
Step1: Cells were pre-treated with O3, Step 2: Cortisol was added and O3 treatment was, depending on the condition either continued or stopped, Step 3: Differentiation was started, cortisol continued and O3 treatment depending on condition either continued or stopped before cultures were fixed. O3: DHA, EPA, RSVL; Cort: Cortisol; Pro: Proliveration; Diff: Differentiation

2.1.6 Modulation of PPARγ activity

To assess whether the effects of RSVL on proliferation and differentiation are mediated via the transcription factor PPARγ and the gene *Klotho*, cells were treated with the PPARγ activator Rosiglitazone or with the irreversible PPARγ blocker GW9662 or with specifically designed siRNAs binding *Klotho* mRNA to knock down *Klotho* expression. The drugs (Table 2-5) were dissolved in 100% Dimethyl sulfoxide (DMSO) and diluted in growth medium. See Table 2-5 for final concentration of compounds. The final DMSO concentration was 0.003% and control cultures were treated with an equivalent concentration of DMSO. Cells were seeded in 96 well plates for immunocytochemistry as described before and cultured for 24h in growth medium, before starting treatment. To assess whether RSVL exerts its effect on AHN via *Klotho*, *Klotho* expression was knocked down 24h after seeding. After 24h under proliferation conditions RSVL or Rosiglitazone (ROSI), a PPARγ agonist, were added.

To assess whether the effects of RSVL on proliferation and neurogenesis are mediated via the transcription factors PPARγ and Klotho, HPC03A/07 cells were treated with the specific PPARγ agonist Rosiglitazone or with the irreversible PPARγ antagonist GW9662 or with siRNAs binding *Klotho* mRNA to knock down Klotho protein expression. Rosiglitazone belongs to the class of drugs of thiazolidinediones that acts by activating PPARs. Rosiglitazone specifically -and with high affinity (K_d=40nM) targets and activates PPARγ (Lehmann et al., 1995).

GW9662 is an irreversible PPARγ antagonist that binds PPARγ with IC50 in nanomolar range (5.4±0.6 nM), and is 10- and 600-fold less potent in binding PPARα and PPARδ, respectively (Leesnitzer et al., 2002; Seimandi et al., 2005). GW9662 was therefore used at 80 nM to avoid blocking of PPARα and

PPARδ. The functional activity of GW9662 as an antagonist of PPARγ was confirmed in an assay of adipocyte differentiation (Lehmann et al., 1995; Braissant et al., 1996). The antagonistic effect of GW9662 was also measured by the inhibition of CD36 expression in peritoneal macrophages stimulated by various ligands, including Rosiglitazone. It further maintains its activity in tissue culture (Huang et al., 1999; Leesnitzer et al., 2002).

For the proliferation assay cultures were grown for 3 days under proliferation conditions. (Figure 2-10 1.)) For the differentiation assay, differentiation was started at the same time as RSVL or ROSI were added and grown for 7 days under differentiation conditions as shown in Figure 2-10, 2.) and Table 2-4, conditions 1 and 2.

After 24h under proliferation conditions cells were then treated with RSVL or ROSI and maintained under proliferation conditions for 3 days. For the differentiation assay differentiation was started at the same time as RSVL or ROSI were added and grown for 7 days under differentiation conditions as shown in Figure 2-10 and Table 2-4.

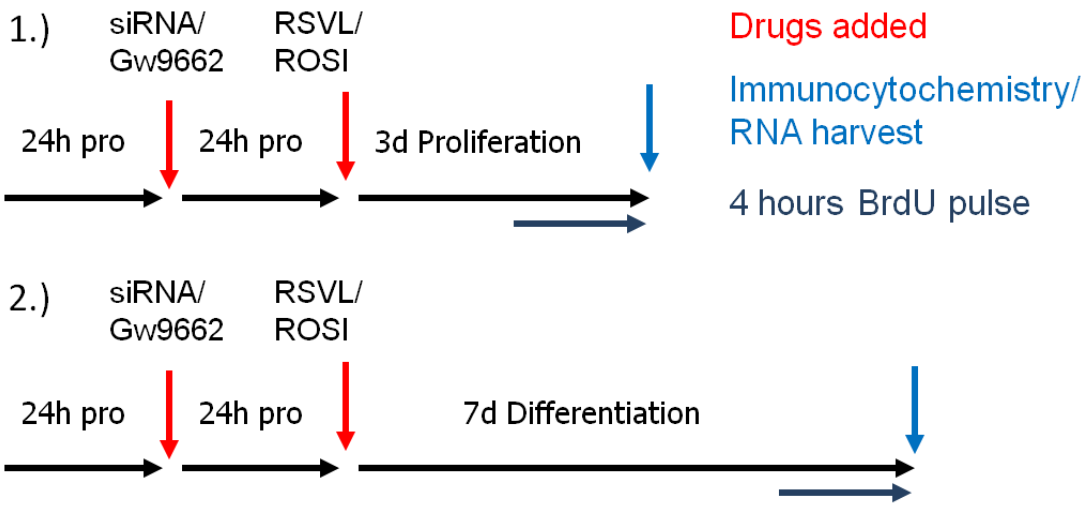


Figure 2-10 Timeline for PPARγ experiment on HPC0A07/03A cells
1.) Proliferation assay, 2.) Differentiation assay. Cells were cultured for 24h after seeding before siRNA or GW9662 were added, after 24h proliferation RSVL or ROSI were added. For the proliferation assay cells were then maintained under proliferation conditions for 3 days, for the differentiation assay differentiation was started simultaneously and maintained for 7 days.

1.	24h pro	24h pro siRNA	7d diff RSVL	5.	24h pro	24h pro RSVL	3d/7d diff RSVL
2.	24h pro	24h pro siRNA	3d/7d diff Rosi	6.	0.003% DMSO		
3.	24h pro	24h pro GW9662 80nM	3d/7d diff RSVL				

Table 2-4 Conditions for PPAR experiment.
Cells were cultured for 24h after seeding before siRNA transfection or GW9662 was added. After 24h RSVL or ROSI was added. For the proliferation assay cell were maintained for 3 days under proliferation conditions. For the differentiation assay differentiation was stared simultaneously and maintained for 7 days. Pro: Proliferation; Diff: Differentiation

Compounds	**Solvent**	**Function**	**Final concentration**
GW9662 (GW)	DMSO	Irreversible PPARγ blocker	80nM
Rosiglitazone (Rosi)	DMSO	PPARactivator	3µM
Resveratrol (RSVL)	DMSO	Stilbenoid targeting PPAR γ	1µM

Table 2-5 PPARγ activator and blocker, function and concentration.
All compounds in this table have been dissolved in DMSO and diluted in media to their final concentration.

2.1.7 RNA interference (RNAi)

To suppress the protein expression of Klotho, cells were transfected with stealth siRNA (Invitrogen) that specifically binds *Klotho* mRNA in the cytoplasm (see Table 2-6 for sequences). The transfection method for HPCOA07/03 cells was already in place in the lab. They were transfected using the N-TER Nanoparticle siRNA Transfection System (5nM) (Sigma N2913) and again BLOCK-iT Alexa Fluor red Fluorescent Oligo (5nM) (Invitrogen) was used to determine the efficiency of transfection which was estimated at >80% (Figure 2-12). Cells were then either fixed for immunocytochemistry (Section 2.2) or harvested for RNA (Section 2.4) or protein extraction (Section 2.3).

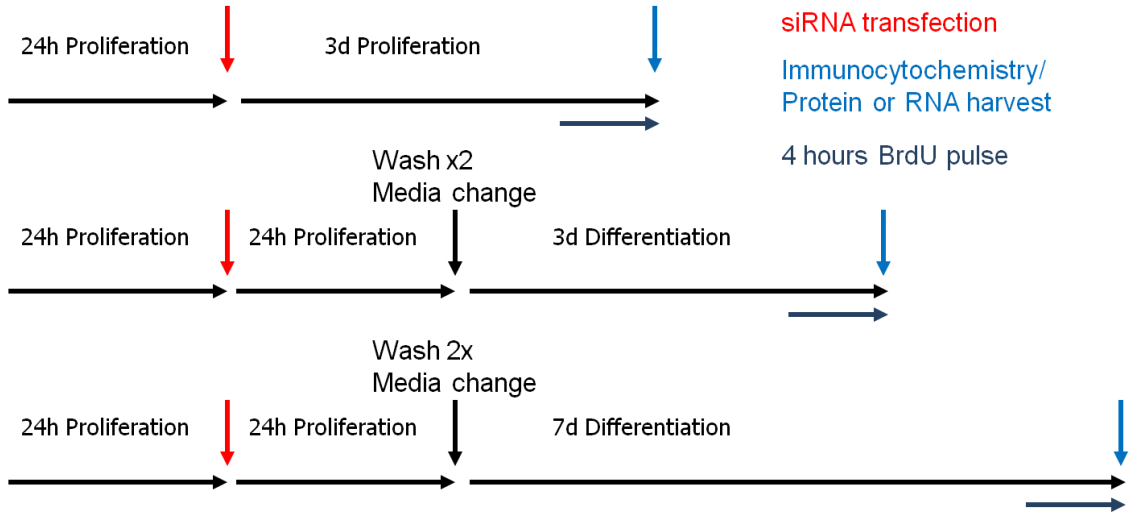


Figure 2-11 Timeline for siRNA transfection of HPCA07/03A cells.
For the proliferation assay (line 1) cells were transfected using the N-TER Nanoparticle siRNA Transfection System 24h after seeding and maintained for 3 days. For the differentiation assay (line 2 and 3) cells were kept for 24h under proliferation was started and maintained for 3 or 7 days.

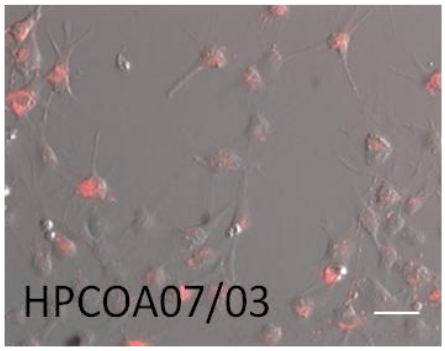


Figure 2-12 HPCOA07/03 after transfection/nucleofection
Cultures were transfected/nucleofected with Block-iT Alexa Flour red Fluorescent Oligo using the N-TER Nanoparticle siRNA Transfection System. Transfection efficiency was >80%.

siRNA sequences used in HPCOA07/03 against human *Klotho*:
CCUGAGGCAACUGCUUUCCUGGAUU AAUCCAGGAAAGCAGUUGCCUCAGG Exon 2
GGACUCUUCUAUGUUGACUUUCUAA UUAGAAAGUCAACAUAGAAGAGUCC Exon 3
CCCGAAAGUCUUUACUGGCUUUCAU AUGAAAGCCAGUAAAGACUUUCGGG Exon 5

Table 2-6 Sequences of stealth siRNA's (Invitrogen) used for the *Klotho* knock down in HPCOA07/03.

2.1.8 Generation of a cell line conditionally over expressing Klotho

To assess the effect of the secreted form of the gene *Klotho* on the proliferation and differentiation of HPC0A07/03A cells, these cells were genetically engineered to conditionally over-expressing the secreted form of *Klotho* using the Lenti-X™ Tet-On® Advanced Inducible Expression System (Clonetech). Gene expression is activated in this system using the tetracycline Doxycycline. The Lenti-X™ Tet-On® Advanced Inducible Expression System consists of a regulator vector: pLVX-Tet-On Advanced, and a response vector: pLVX-Tight-Puro. The regulator vector constitutively expresses a tetracycline-controlled transactivator (rtTA-Advanced) that, in the presence of Doxycycline binds to the inducible promoter (P_{tight}) of the gene of interest in the response vector and activates transcription. P_{tight} consists of a Tet-Responsive Element (TRE) joined to a minimal CMV promoter. Induction of the system produces high-level transcription of the gene of interest. See Figure 2-13.

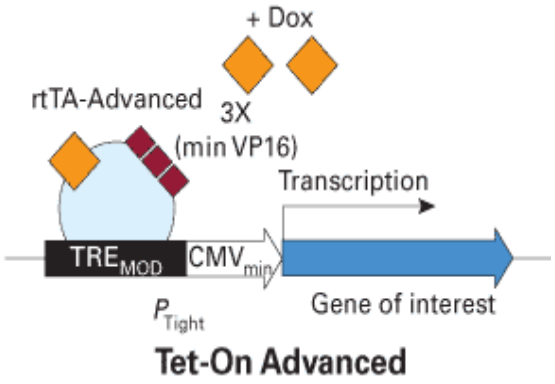


Figure 2-13 Lenti-X™ Tet-On® Advanced Inducible Expression System.
The Lenti-X™ Tet-On® Advanced Inducible Expression System consists of a regulator vector: pLVX-Tet-On Advanced, and a response vector: pLVX-Tight-Puro. The regulator vector constitutively expresses a tetracycline-controlled transactivator (rtTA-Advanced) that, in the presence of Doxycyclin binds to the inducible promoter (P_{tight}) of the gene of interest in the response vector. P_{tight} consists of a Tet-Responsive Element (TRE) joined to a minimal CMV promoter. Induction of the system produces high-level transcription of the gene of interest. Picture source: www.clontech.com.

2.1.8.1 Cloning of Kotho secreted into the pVLX-Tight-Puro vector

The human *Klotho* secreted plasmid in pcDNA3.1/V5/His-TOPO back bone (17713) was purchased from Addgene (Cambridge, USA) and was then cloned into the pLVX-Tight-Puro vector (ClonTech 632162) using the enzymes BamHI and Xba1 (NEB), as follows: The vector and the target gene plasmid were digested using the same enzymes to create matching sticky ends. The cutting site of the enzyme Xba1 (T/CTAGA) is blocked by overlapping dam methylation. Dam methylase–methylation occurs at the N^6 position of the adenine in the sequence GATCT (Marinus and Morris, 1973; Geier and Modrich, 1979). To be able to digest the blocked Xba1 site, the plasmids were transferred into Stellar^{TM} dam$^-$/dcm$^-$ competent cells (Clontech) following manufacturer's instructions to prevent blocking of the site through dam methylation. The plasmids were extracted using the Sigma GenElute miniprep (PCN70-1KT) following manufacturer's instructions. To isolate the specific fractions, vector and insert, the plasmids were then subjected to a double digest with BamHI and Xba1 using 5ug of DNA. The products of the digest were concentrated using QIAprep Spin Miniprep Kit (Qiagen) following manufacturer's instructions, checked for quality on an ethidium bromide gel and separated using a Crystal blue gel. The insert and vector were then purified from the gel using QGbuffer (Qiagen) and the GeneJetPlasmidMiniprep kit (Fermentas) following manufacturer's instructions. For ligation, the insert DNA and the vector DNA were mixed with 1µl ligase (NEB) and 2µl 10x ligation buffer (NEB) in a total reaction volume of 10µl and incubated for 30min at RT. The plasmid was then transformed into dam$^-$/dcm$^-$ competent cells and sequenced for the correct insertion of the target gene as described in section 2.1.8.2. The plasmid was then extracted and purified using PureYield Plasmid Maxiprep (Promega)

following manufacturer's instructions. A glycerol stock of the E.Coli carrying the *Klotho* secreted pLVX-Tight-Puro Plasmid was stored at -80°C.

2.1.8.2 Sequencing of the Klotho secreted pVLX-Tight-Puro plasmid

The plasmid was amplified using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) using the primers in Table 2-7 and the conditions in Table 2-8 on the BioRad Opticon 2. To remove unincorporated nucleotides, the sequencing PCR product was cleaned up using the Ethanol/EDTA precipitation protocol following manufacturer's instructions (Applied Biosystems), resuspended in Hi-Di Formamide (Applied Biosystems) and assayed on a Applied Biosystems 3130 genetic analyzer. The resulting sequence electropherograms were then analysed using BioEdit and CodonCode Aligner software.

Forward primer	
CMV	CGCAAATGGGCGGTAGGCGTG
Fw1	AGGCCCTTTCGTCTTCACTC
Fw2:	TAGCCAGCGACAGCTACAAC
Fw3	CATCGACAACCCCTACGTG
Reverse primer	
BGH	TAGAAGGCACAGTCGAGG
Rev1:	GGGGAACTTCCTGACTAGGG
Rev2:	TAGGGCTTGGTGAGACTGCT
Rev3	GGTCCAAAGCAAAGAGCAAA

Table 2-7 Sequencing primer:
Primer used for sequencing the correct insertion of the *Klotho* secreted insert into the pVLX-Tight-Puro vector

96.0°C 01:00 min
96.0°C 00:08 min
50.0°C 00:07 min
60.0°C 01:30 min
Go to line 2 for 14 more times
96.0°C 00:08 min
50.0°C 00:07 min
60.0°C 01:45 min
Go to line 6 for 4 more times
96.0°C 00:08 min
50.0°C 00:07 min
60.0°C 02:00 min
Go to line 10 for 4 more times
10.0°C 16:00:00
End

Table 2-8 PCR protocol used for amplifying the *Klotho* secreted pLVX-Tight-Puro Plasmid

2.1.8.3 Lentivirus generation

For the transfection of the HPC03A/07 the modified Lenti-X™ Tet-On® Advanced Inducible Expression System (Clonetech) has been used. To generate and package this virus the envelope plasmid pMDG: VSV-G and the packaging plasmid P8.91: GAG-POL was used instead of the Lenti-X HT packaging mix, and the transfection was conducted using Lipofectamine instead of Lentiphos HT, as this set up was already in place in the lab. To generate the two viruses, one with the regulator vector and one with the response vector, HEK cells were transfected using Lipofectamine following manufacturers' instructions with a total of 30µg of DNA containing the envelope plasmid pMDG:VSV-G, the packaging plasmid P8.91:GAG-POL (provided by Graham Cocks, IOP, King's College London, U.K.) and either the regulator or the response plasmid. The virus was collected 24h and 48h after the transfection and concentrated using the Lenti-X concentrator (Clontech) following manufacturers' instructions. The concentrated virus was stored in aliquots at -80°C. See Figure 2-14 for an overview of the lentivirus generation and cell transduction.

2.1.8.4 Transduction of the HPC03A/07 cell line using Lentivirus

HPC03A/07 cells were cultured for 48h as described previously, media was changed 24h before the viral transduction. Cultures were transducted in 6 well plates (nunc) at 60% confluency. 5µl of regulator virus and 10µl of response virus were added to 2ml of media and centrifuged for 45min at 750g, 32°C. After 30min incubation at 37°C the media was changed and 48h later the cells were subjected to antibiotic selection using 0.2µl/ml Puromycin. The *Klotho* secreted pVLX-Tight-Puro expresses resistance to the antibiotic Puromycin. To ensure only successfully transducted cells were cultured further the cells were

subjected to Puromycin for 2 days. Cells not carrying the plasmid were dead after 2 days. The pLVX-Tet-On-Advanced plasmid expresses resistance to the antibiotic Neomycin; however the HPC03A/07 cells are already resistant to Neomycin after they have been made conditionally immortalised with the c-myc-ER. None the less the successful transduction of both plamids was confirmed by immunocytochemistry against the secreted form of Klotho, the gene of interest. The protein of the gene of interest can only be expressed if the cell carries both plasmids, the regulator and the response vector. Cells, further referred to as Klover, were then passaged into a T75 flask (nunc) and cultured or frozen as needed for the experiment.

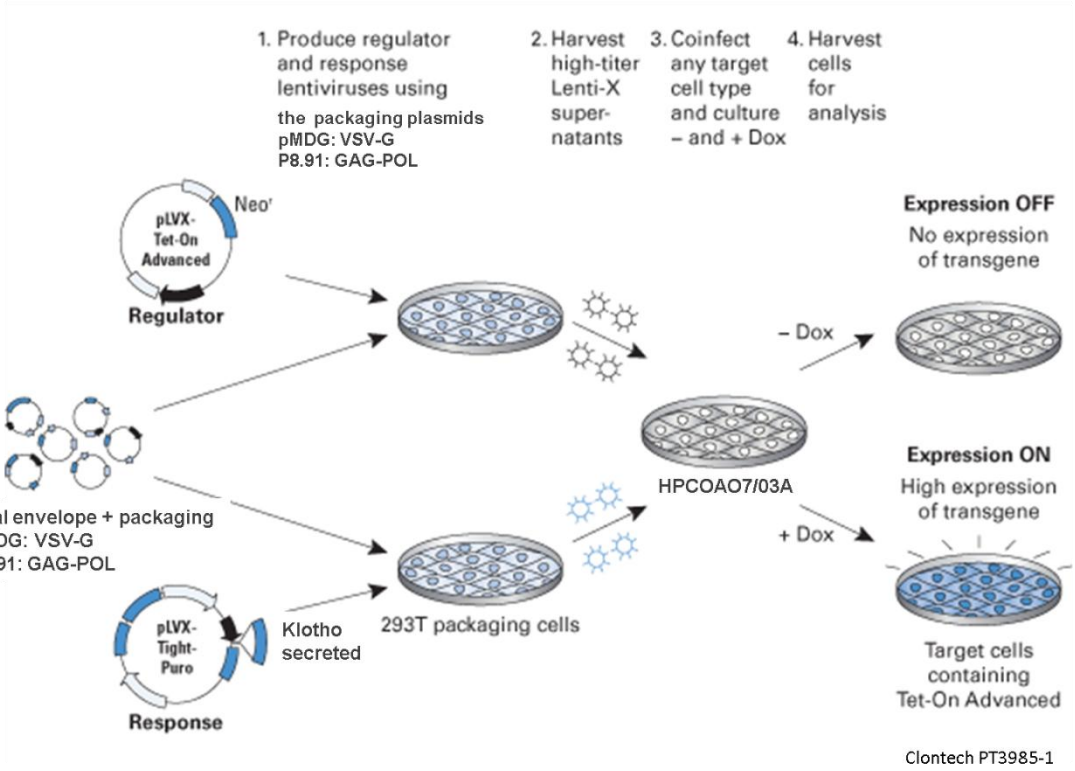


Figure 2-14 Establishing an inducible expression system in the HPC03A/07 cell line with Lenti-X TetOn Advanced:
1. The response and the regulator lentivirus were produced using the enveloping plasmid pMDG: VSV-G, the packaging plasmid P8.91: GAG-POL and 293T cells. 2. Virus was harvested in the supernatant of the cell culture. 3. HPCOA7/03A cells were coinfected with to two viruses. 4. Cells were harvested for experiments Picture slightly modified from www.clontech.com

2.1.8.5 Klover experiment

To induce expression of the secreted form of *Klotho* in the Klover HPC03A/07 cells, the cultures were treated with Doxycycline (Sigma) (1µg/ml) 24h after seeding. For the proliferation assay cells were cultured for 3 days under proliferation conditions, for the differentiation assay differentiation was started at the same time as *Klotho* expression was started by adding Doxycycline (Figure 2-15).

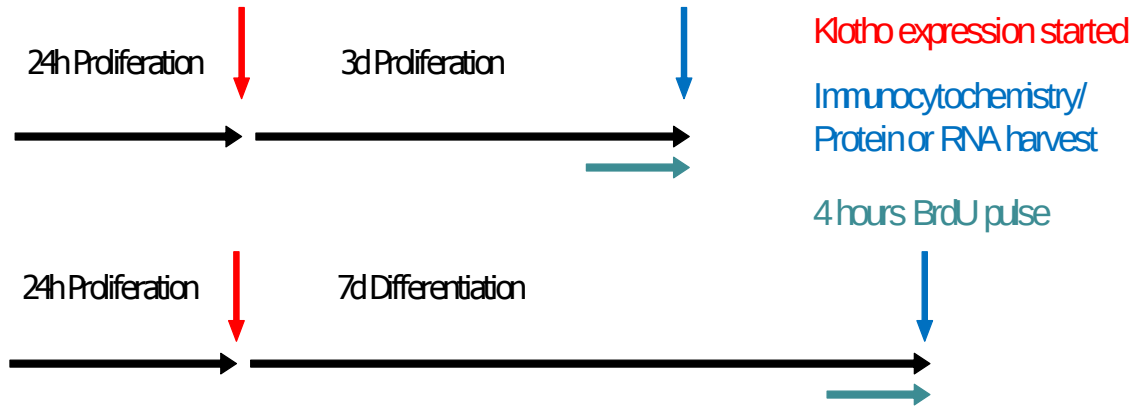


Figure 2-15 Timeline for the experiment using Klover HPC03A/07 cells
Cells were treated with 1µg/ml Doxycycline 24h after seeding. Cultures were then grown under proliferation conditions for 3 days or under differentiation conditions for 7 days.

2.1.9 Analysis of cell proliferation

The thymidine analogue 5'-bromo-2'-deoxyuridine (BrdU) is commonly used to assess cell proliferation as the synthetic nucleotide incorporates into the DNA of cells undergoing DNA replication during S-phase. BrdU at 10µM has been added for 4h, the time of the S-phase, before fixation. Staining for incorporated BrdU with commercially available antibodies is used to investigate the rate of cell proliferation (Wojtowicz and Kee, 2006).

For BrdU detection via antibodies the DNA needs to be denaturised so the antibody gains access to the DNA-integrated BrdU (Kee et al., 2002). This was achieved by incubating the fixed cells in 2N Hydrochloric acid HCL for 15min at RT. Subsequently cells were rinsed with PBS three times for neutralisation. The staining was then carried out as described in section 2.2 below.

Proliferating cells were also identified using the marker Ki67. Ki67, a nuclear protein expressed throughout the cell cycle phases except during the G_0 and early G_1 phase, is a reliable marker for proliferation that is expressed exclusively during mitosis and has a very short half-life. (Kee et al., 2002)

2.2 Immunocytochemistry

A range of antibodies (Table 7-3) were used to assess the proliferation, differentiation and survival of progenitor cells after they have been exposed to various compounds.

To overcome non-specific background staining of the secondary antibodies that were raised in Donkey, cultures were blocked with PBS containing 5% Normal Donkey Serum (NDS) and 0.1% Triton-X-100, for 1h at RT and then incubated with one or two primary antibody (Table 7-3) over night at 4°C. The next day

cultures were rinsed three times with PBS, blocked for 30min at RT, incubated with the appropriate secondary antibody (Table 7-4) for 2h at RT. See Table 7-5 and Table 7-7 for detailed combinations of primary and secondary antibodies. For a nuclear counter stain cultures were rinsed in PBS containing 300nM 4',6-diamidino-2-phenylindole (DAPI) (Sigma) for 2min followed by two PBS washes. DAPI binds strongly to DNA of fixed and live cells. Finally, cultures were either, in case cells were grown on glass objective slides using the reusable chamber slides, mounted with cover slips and ProLong® Gold (Invitrogen) or, in the case of 96 well plates, stored in 200µl PBS plus 0.05% Sodium azide (to prevent bacterial growth) at 4°C. As a negative control fixed cultures underwent the same treatment as above but without adding the primary antibody.

BrdU staining was also conducted, however because of technical issues the data could not be presented in this thesis.

2.3 Quantitative protein analysis

2.3.1 Protein extraction

In order to relatively quantify the amount of Klotho protein in the Klover cells and the HPC03A/07 after *Klotho* knock down using siRNA, cells were lysed into ice cold modified RIPA lysis buffer (Table 7-8) containing protease and phosphatase inhibitors (Sigma/ Roche), constantly agitated for 30 minutes at 4°C before being centrifuged in a microcentrifuge at 4°C (12,000g, 20min). The supernatant was transferred into a fresh tube and stored at -80˚C. Protein concentration was determined using the BCA Protein Assay Kit from Novagen (71285-3) according to manufactureres instructions.

2.3.2 Protein separation and detection

Samples were thawed at 4˚C before being boiled at 90˚C for 5 minutes to denature proteins. A total of 5ug of protein was loaded on a reducing 10% SDS-PAGE gel (Table 7-9) for 1 hour at 200V. Proteins were then electrophoretically transferred to a nitrocellulose membrane, 0.2 µm pore size (Invitrogen) at 25V for 1.5h at RT. Transfer efficiency was controlled by Precision Plus Protein Dual Color Standards (BioRad). Unspecific binding sites were blocked for 1h in 4% non-fat dry milk in TBS and immunoprobed with the KM2076 antibody (Kyowa Hakko Kirin) against Klotho's secreted form (expected band size 60kD) in background reducing antibody diluents (DAKO) at 4°C overnight. The next day, membranes were washed three times with TBS containing 0.1% Tween-20 (TBS-Tween) and incubated with a Alexa-Flour Goat anti rat 680 secondary antibody (Invitrogen, 1:3000) in 4% non-fat dry milk in TBS for 1h at RT before being washed three times in TBS-Tween. Proteins were visualised using the Odyssey detection system. The relative density of the Western blot bands was analysed using ImageJ software.

2.4 Quantitative RNA analysis

2.4.1 RNA extraction

RNA was extracted from cell lines and rat brains using the TRI Reagent (Ambion) procedure according to manufacturer's instructions. Samples were stored at -80°C.

2.4.2 DNAs treatment

To remove genomic DNA from the sample it was treated with the DNA-free™ DNase Treatment and Removal Reagents (Ambion) according to manufacturer's instructions. RNA was quantified using spectrophotometry

(NanoDrop) and the purity determined via the 260/230 ratio to assess quality, clean RNA for the following cDNA synthesis. Only RNAs with a ratio above 1.80 were included.

2.4.3 cDNA synthesis

cDNA synthesis was completed with the Superscript III (Invitrogen) using 1µg of RNA and random decamers (Sigma) at 250ng, as described by the manufacturer. The parameters used for the PCR reaction are given below. The samples were dilute 1:10 with DEPC water and stored at -20°C.

Denaturing
65ºC for 5min
Place straight on ice for ~1min

RT Protocol

25 ºC	5 min
42 ºC	120min
70 ºC	15min
4 ºC	60min

2.4.4 Quantitative PCR

qPCR was used to detect and ultimately quantify the expression level of the gene *Klotho* and PPARγ. See Table 2-9 for details.

Genes of interest			
Klotho sec	232bp	>F TTGGAATCTCCCAACCTGAG	
		>R ACTCGAAACCATCCATGAGG	Exon2-3
PPARγ	136bp	>F CTCCGTGGATCTCTCCGTAA	Exon2
		>R TGCAACCACTGGATCTGTTC	
Housekeeping genes			
UBC	133bp	>F ATTTGGGTCGCGGTTCTTG	
		>R TGCCTTGACATTCTCGATGGT	
HPRT1	94bp	>F TGACACTGGCAAAACAATGCA	
		>R GGTCCTTTTCACCAGCAAGCT	
bACT	140bp	>F CTGGAACGGTGAAGGTGACA	
		>R AAGGGACTTCCTGTAACAATGCA	

Table 2-9 Q-PCR primer
Sequences, length of PCR product and binding area of the primer used in this thesis.

qPCR quantifies the gene copy number through continuous monitoring of a fluorescent signal that is only emitted if the fluorescent dye is bound to a double stranded product generated during PCR. This fluorescent signal is proportional to the DNA concentration. For all qPCRs the BioRad Opticon 2 has been used (Protocol below).The master mix contained 4µl 5xHOT FIREPol EvaGreen qPCR Mix (Solis BioDyne), 4µl of 1µM forward and reverse primer at a final concentration of 100nM and 8µl of water. The final reaction volume was made up of 16µl Master Mix and 4µl cDNA (1:10). The primers, all custom made by Sigma or IDT, were tested for primer dimers and the concentration optimized using cDNA from human or rat hippocampus.

Q-PCR cycle

1	95ºC	00:15:00
2	95 ºC	00:00:30
3	60 ºC	00:00:30
4	72 ºC	00:00:30
5	see below	00:00:10
6	Plate read	
7	Go to line 2 for 44 more times	
8	Melting curve: from 60 ºC-95 ºC, read every 1 ºC and hold for 10min	

Step 5:	Normal:	80.0 ºC
	FGF23:	80.5 ºC
	Wnt3a:	81.5 ºC
	MAPT:	82.0 ºC

2.5 Immunohistochemistry on free floating rat brain sections

These experiments were carried out to evaluate the functionality of the Klotho antibody and to find out which cell types express *Klotho*.

2.5.1 Fluorescence staining

Control mouse brain sections were co-stained for Klotho protein and markers for proliferation SOX2, markers for differentiation NEUN and the glial marker GFAP to see which cell types co-express Klotho. See Table 7-3. Sections were stained free floating using net carriers in 6-well plates. First, the cryoprotective solution was rinsed off with PBS on a shaker for 5min. This wash was repeated three times. Sections were then blocked with 3% Normal Donkey serum in PBS with 0.25% Triton-X for 60min at RT to prevent unspecific binding of the secondary antibody that was raised in Donkey. Sections were then incubated with primary antibodies overnight at 4^0C on a shaker. The next day the sections

were given three 5min washes in PBS, blocked for 30min and then incubated with the appropriate secondary antibody (Table 2-10) for 2h at RT. Finally sections were rinsed in DAPI for 5min, twice in PBS and then mounted on glass slides using ProLong® Gold (Invitrogen). As negative control a section underwent the same treatment as described above however without adding a primary antibody.

Primary antibodies	Secondary antibodies
KM2119 (rat) Ki67 (rabbit)	Anti rat 488 Anti rabbit 594
KM2119 (rat) NEUN (mouse)	Anti rabbit 488 Anti mouse 594
KM2119 (rat) GFAP (rabbit)	Anti rabbit 488 Anti mouse 594

Table 2-10 Primary and secondary fluorescent AB combinations for sections

2.5.2 3,3'-Diaminobenzidine (DAB) staining for stereology

Sections (1:6) for stereology of the IF mouse model (explained in detail in 2.6.1.2) were stained for the proliferation marker Ki67, Dcx, a marker for early neurons and Klotho (SC) (Table 2-11) to explore any differences in their expression in the hippocampus of these mice.

Klotho (E-21)	goat	Santa Cruz BT	SC-22220	1:500
Ki67	rabbit	abcam	Ab15580	1:500
Dcx	rabbit	abcam	Ab18723	1:10000

Table 2-11 Primary antibodies used for DAB staining

Sections were incubated in 1% H_20_2 in TBS for 40 minutes at RT to quench endogenous peroxidase activity. Next, sections were rinsed in TBS before being blocked in 15% normal serum (NS) in TBS-T (TBS + 0.3% Triton-X) for 30 min. Incubation with primary antibodies (Table 7-3) was carried out in a dilution of 10% NS in TBS-T at 4°C overnight. The following day sections were rinsed in TBS and incubated with the appropriate biotinylated secondary antibody diluted in 10% NS in TBS-T for 2h. Sections were then rinsed again before an avidin-biotin-peroxidase complex, diluted at 1:1.000 in TBS, was applied for 2h (Vectastain Elite ABC kit, Vector Laboratories). Finally the staining was visualised by a standard DAB reaction (0.05% 3,3'-diaminobenzidine tetrahydrochloride HCl; Sigma) for 5-15 minutes, before sections were rinsed and mounted onto chrome-gelatine coated slides, dried overnight, cleared and dehydrated in 100% IMS and 100% xylene before being coverslipped with DPX (VWR).

As a negative control, sections were treated following the protocols above but instead of the primary antibody they were incubated in PBS and then incubated with secondary antibody.

2.6 Microscopy

2.6.1.1 Fluorescence

Pictures of immunocytochemically stained cells were taken on a fluorescence microscope Axio Imager microscope (Carl Zeiss Inc.) using the Axio vision Digital Image Processing Software Version 4.67.1 (Carl Zeiss Inc.). Bright field images and images of immunocytochemically stained cells in 96 well plates were taken with the Olympus 1x70 inverted microscope using the same imaging software. Pictures were imported into ImageJ for further processing.

2.6.1.2 Stereology

Stereology is a tool used to obtain quantitative properties about structure, regional volumes, cell numbers and other biological parameters of 3D objects from 2D sections through an object (Schmitz and Hof, 2005). Stereology has become the gold standard for the analysis of quantitative information in the CNS. The random, systematic sampling guarantees the collection of unbiased, accurate results from small sample sizes with statistical efficiency. To assure equal sampling, a series of regularly spaced sections is required spanning the whole area of interest (Gundersen and Jensen, 1987). A grid of known spacing is then applied and provides a series of systematic sampling sites. In this study murine sections of 40µm thickness were used to quantify the amount of Klotho expressing cells in the DG. The randomly but systematic applied grid provides an unbiased dissector frame through which small regions of the area of interest are quantified independent of orientation, size and shape of the analysed objects (Gundersen et al., 1988; Molteni et al., 2002). The sizes of the sampling sites and the dissector frame are predetermined according to the size of the region of interest and the number of sections was kept constant throughout the

analysis of the experiment in order to produce a Gundersen coefficient of error of less than 0.1. The parameter for the grid size in this study were X: 234.01µm, Y: 80.81µm and 37µm x 37µm for the counting frame. Throughout the study the x40 objective was used for counting.

All stereological analyses were performed using *StereoInvestigator* software (Microbright Field, Williston, VT) on a Zeiss Axioskop2 MOT microscope (Carl Zeiss Ltd) and a DAGE-MTICCD-100 camera (DAGE-MTI Inc.). The optical fractionator method was used to quantify Klotho positive cells in the Dentate Gyrus. It uses thick sections and estimates the total number of cells from the number of cells sampled with a Systematic Randomly Sampled (SRS) set of unbiased virtual counting spaces covering the entire region of interest with uniform distance between unbiased virtual counting spaces in directions X, Y and Z (Gundersen et al., 1988). Analysis was carried out as previously described by (Thuret et al., 2009) with a randomly selected starting section followed by every sixth section thereafter.

2.6.1.3 Threshold analysis

For quantitative threshold image analysis of Klotho immunoreactivity in the DG, different sections were DAB stained simultaneously. Ten non-overlapping images were captured from three consecutive sections per animal. Images were obtained, blind to genotype, with a live video camera (JVC, 3CCD, KY-F55B), mounted onto a Zeiss Axioplan microscope using a 5X objective. Parameters including lamp intensity, video camera setup and calibration were kept constant throughout the analysis. Subsequently images were analysed using the *Image-Pro Plus* 4.0 (Media Cybernetics) image analysis software. For this analysis an appropriate threshold was applied that selected foreground immunoreactivity

above background, which was applied as a constant for all images analysed. The recorded data was transferred to an Excel spreadsheet, analysed statistically and displayed as the mean percentage area of immunoreactivity per field (± SEM).

2.7 Statistical analysis

Data are presented as mean ± SEM. All statistical analyses were performed with GraphPad Prism 5 on independent biological replicates (indicated as n). The number of biological replicates was three if not otherwise stated. One-Way ANOVA with Newman-Keuls post hoc test was used for multiple comparisons among treatment groups. Student's *t*-test was used to compare means of two independent treatment groups. P-values <0.05 were considered significant. P-values 0.01 – 0.05: *; P-values0.001 – 0.01: **; P-values <0.001: ***,
P-value <0.0001: ****.

Chapter 3 Effects of Omega-3 fatty acids and Resveratrol on HPC03A/07

In this chapter I investigated the effect of Omega-3 fatty acids and RSVL on hippocampal neurogenesis in an *in vitro* model of hippocampal neurogenesis using the human embryonic hippocampal progenitor cell line HPC03A/07, specifically I have:

Investigated the effects of the Omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the stilbenoid Resveratrol (RSVL) on HPC03A/07 during proliferation and differentiation.

Determined the possible preventative properties of Omega-3 and RSVL in an *in vitro* model of stress. The model used the HPC03A/07 cells treated with Cortisol to mimic stress resulting in a decrease in proliferation and neurogenesis (see Section 2.1.5.1 for details).

3.1 EPA and DHA decrease apoptosis and increase the proportion of dividing cells and neuronal cells in HPC03A/07

In order to determine the effects of EPA or DHA on proliferating and differentiating HPC03A/07 cells, cells were cultured as described in Section 2.1.1. The concentration of 10µM was determined previously in the Thuret lab by titration (data not shown) and did prove to result in the highest increase in proliferation. After 24h under proliferating conditions EPA or DHA treatment was started and for the proliferation assay cells were maintained for further 6 days under proliferation conditions before being fixed with 4% PFA. Media was changed after 3 days. For the differentiation experiment, cell differentiation was started after 6 days of proliferation. Cultures were maintained for 7 days before being fixed for immunocytochemistry. See Figure 2-9. Cultures were then stained for the proliferation marker Ki67 and the apoptosis marker activated Caspase-3. Cultures were double labelled for the NPC marker SOX2 and

Nestin. In the differentiation experiment cells were also stained for the neuroblast marker Dcx and the mature neuronal marker MAP2.

3.1.1 Eicosapentaenoic acid increases the proportion of dividing cells and decreases apoptosis in proliferating HPC03A/07

In proliferating HPC03A/07 cells treated with EPA the percentage of Ki67 positive cells was significantly increased by 13.7±1.2%, $p<0.001$ compared to vehicle treated cells to a total of 39.3±0.4% (Figure 3-1a,b). The percentage of cell expressing activated Caspase-3, however, was significantly decreased by -40.1±11.7%, $p<0.05$ compared to control to a total of 5.4±1.1% (Figure 3-1c,d). The percentage of cells expressing SOX2 or Nestin expression showed no changes.

EPA increased the proportion of dividing cells and decreases apoptosis in proliferating HPC03A/07, suggesting an increased survival of dividing cells.

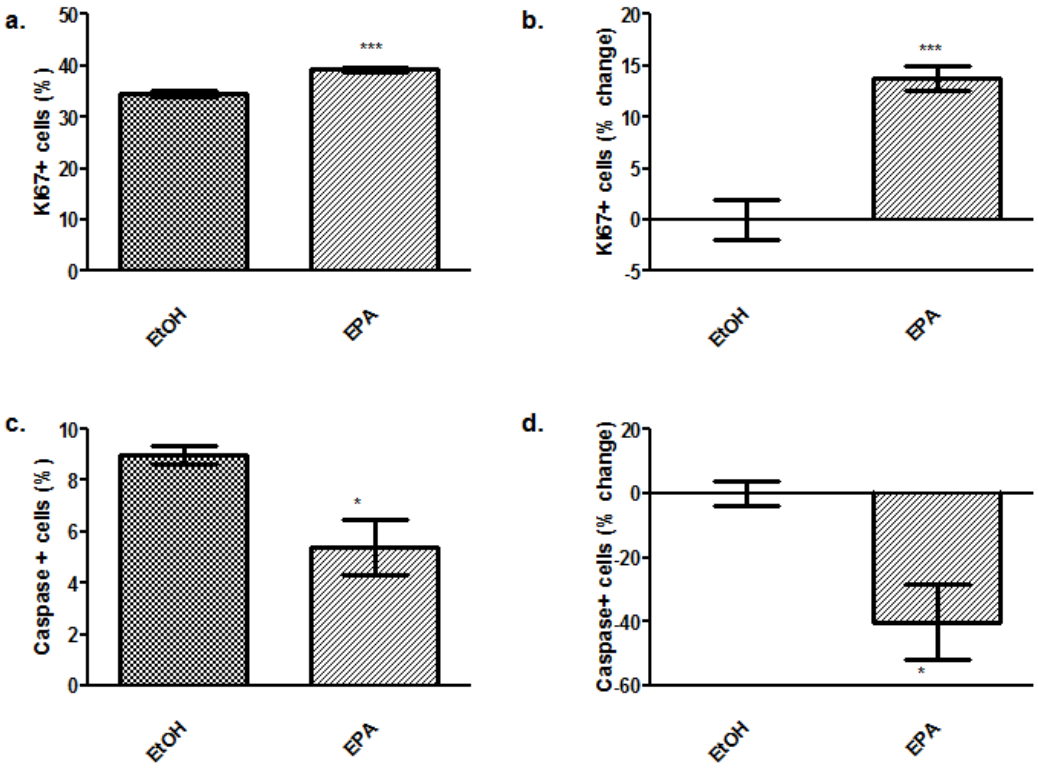


Figure 3-1 Ki67, activated Caspase-3 in proliferating HPC03A/07 treated with EPA

a, c, e, g depict the percentage of positive cells relative to absolute cells. b, d, f, h show the percentage change of the marker in EPA treated cultures compared to EtOH (vehicle) treated cells. a) and b) Ki67 expressing cells c) and d) activated Caspase-3 expressing cells. Abbreviations: EtOH: Ethanol control; EPA: eicosapentaenoic acid (p-values were generated using Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, ***p<0.001).

3.1.2 Eicosapentaenoic acid increases the proportion of dividing cells and of mature neuronal cells and decreases apoptosis in differentiating HPC03A/07

To assess the effect of EPA during differentiation, HPC03A/07 cells were treated with EPA as described in Section 3.1. The percentage of Ki67 positive cells was significantly increased by 21.8±1.1%, $p<0.05$ when EPA cultures were compared with EtOH treated controls to a total of 33.6±0.3% (Figure 3-2a,b). No change was observed in the proportion of Dcx expressing cells present under experimental compared to control conditions (total percentage 16.3±0.5%, Figure 3-2c,d). However, the percentage of MAP2 expressing cells was significantly increased by 45.0±0.9%, $p<0.0001$ to a total of 28.2±0.2% (Figure 3-2e,f). While the percentage of cells labelled with activated Caspase-3 was significantly decreased by -44.2±8.9%, $p<0.01$ to a total of 7.9±1.3% (Figure 3-3a,b). SOX2 showed no changes (Figure 3-3c,d) whereas the percentage of Nestin expressing cells was decreased by -1.8±0.3%, $p<0.01$ to a total of 98.0±0.3% (Figure 3-3e,f).

These data suggest that EPA still increases the percentage of dividing cells in HPC03A/07 after 7 days of differentiation but also increases differentiation along a neuronal pathway, resulting in an increased proportion of ‘mature’ MAP2 expressing neurons but not immature DCX expressing neurons. It further significantly decreased the percentage of apoptotic cells and Nestin expressing cells. This suggests that EPA treatment facilitates a fast differentiation into maturing neurons and enables their survival. Further, the continuing division of some might be due to asymmetric division, meaning cells divide and one daughter cells starts differentiation (in this case into neuronal cells) the other

keeps her progenitor cell properties (here Ki67 positive cells) and continues to divide.

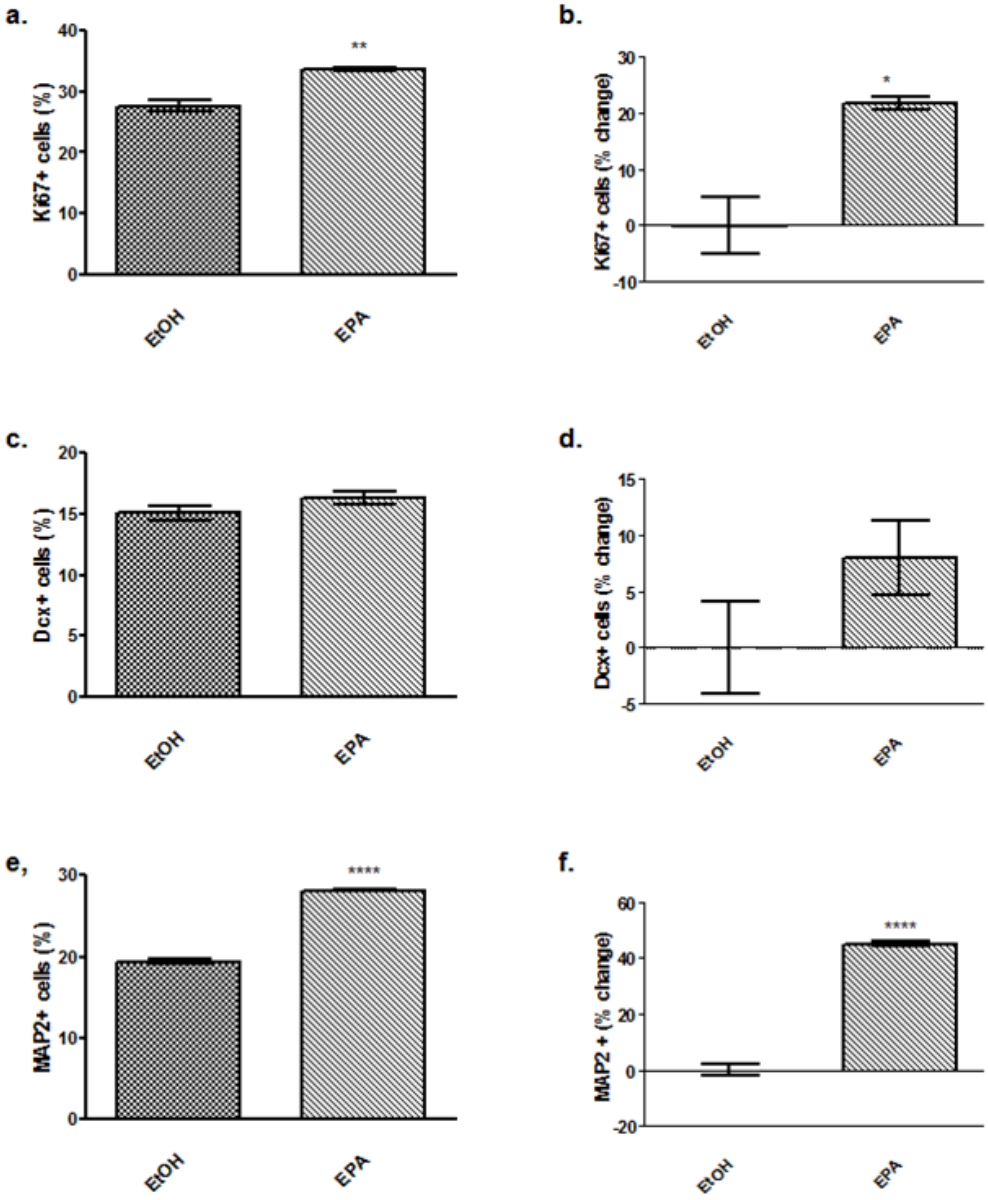


Figure 3-2 Ki67, Dcx and MAP2 expression in differentiating HPC03A/07 under EPA treatment

a, c and e show the percentage of Ki67, DCX and MAP2 expressing cells respective in EPA treated and control cultures. b, d and f show the percentage change of each marker in EPA treated cultures compared to control EtOH treated cells. EPA treatment results in a significant increase in Ki67 expressing cells (a,b) and MAP2 expressing cells (e,f) but no change in DCX expressing cells (c,d). Abbreviations: EtOH: Ethanol control; EPA: eicosapentaenoic acid (p-values were generated using Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ****p<0.0001)

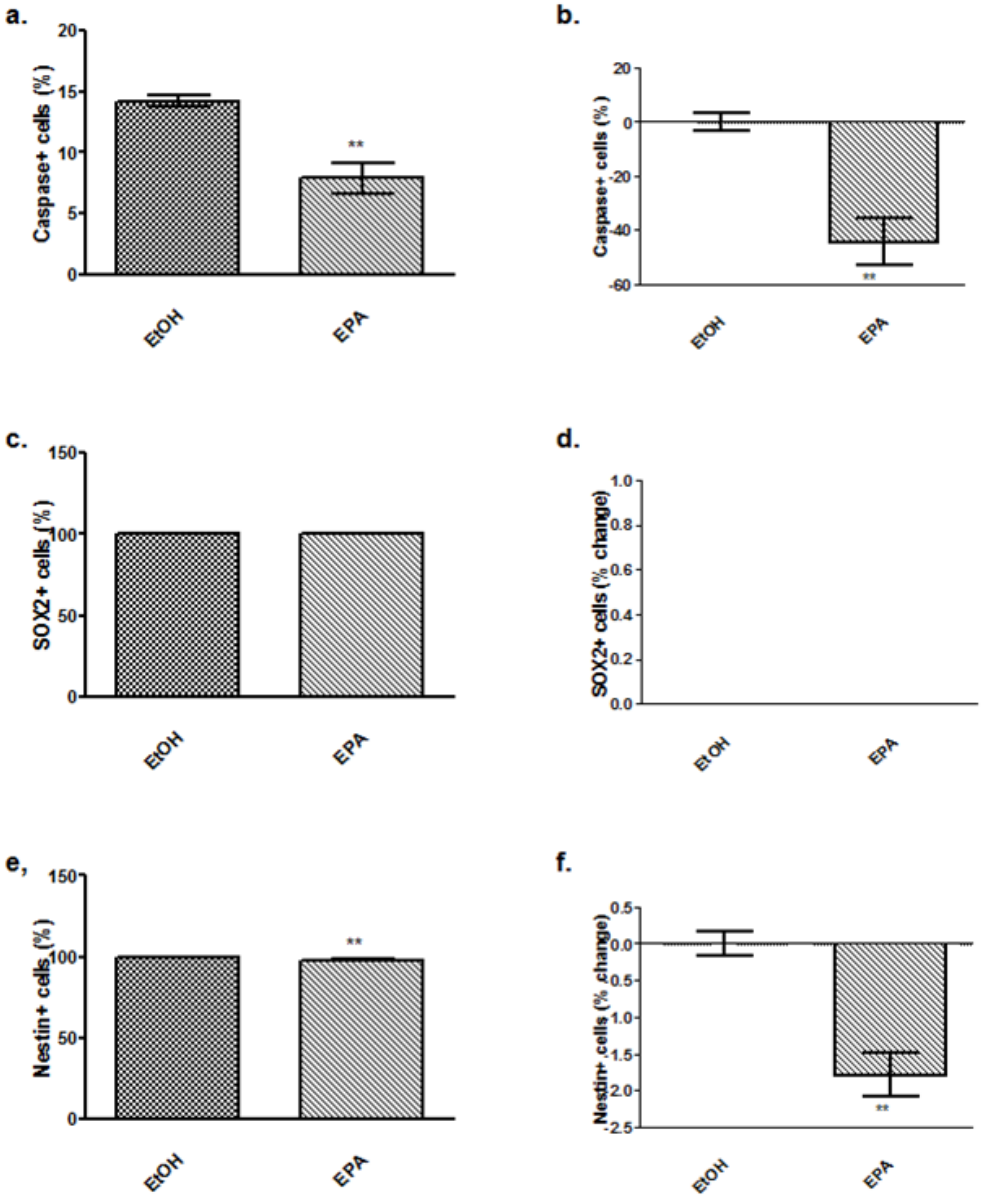


Figure 3-3 Activated Caspase-3, SOX2 and Nestin expression in differentiating HPC03A/07 under EPA treatment

a, c and e show the percentage of activated Caspase-3, SOX2 and Nestin expressing cells respective in EPA treated and control cultures. b, d and f show the percentage change of each marker in EPA treated cultures compared to control EtOH treated cells. EPA treatment resutls in decreased activated Caspase-3 (a,b) and Nestin expression (e,f), but no change in SOX2 expressing cells (c,d). Abbreviations: EtOH: Ethanol control; EPA: eicosapentaenoic acid (p-values were generated using Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), **p<0.01)

3.1.3 Docosahexaenoic acid increases the proportion of dividing cells and decreases apoptosis in proliferating HPC03A/07

Cultures were treated with DHA as described in Section 3.1. In proliferating HPC03A/07 cells the percentage of Ki67 positive cells was significantly increased by 30.0±4.3%, $p<0.01$ compared to EtOH treated control cultures to a total of 51.3±1.2%, (Figure 3-4a,b). The percentage of activated Caspase-3 expressing cells did not significantly change (total of 8.2±0.6%), (Figure 3-4c,d). SOX2 or Nestin expression in DHA treated cultures were unchanged compared to vehicle.

These results indicate that DHA significantly increases the percentage of dividing cells in proliferating HPC03A/07, without impacting on apoptosis and progenitor cells, suggesting that it increases the percentage of dividing cells without affecting survival, therefore DHA might affect proliferation.

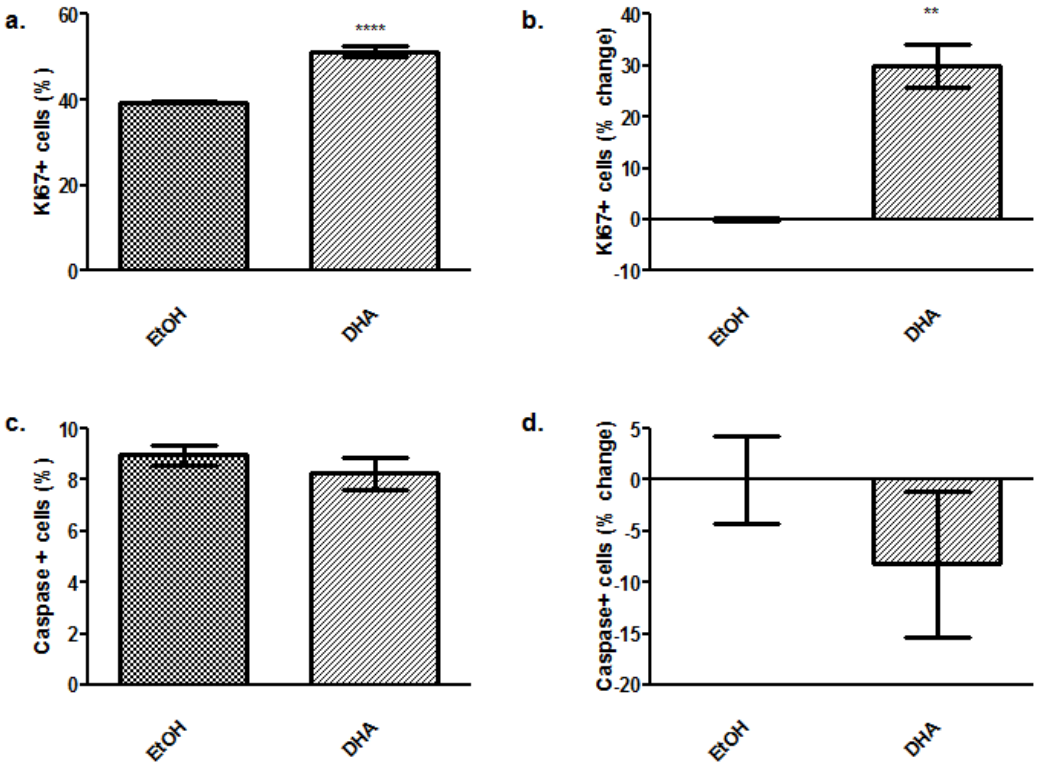


Figure 3-4 Ki67 and activated Caspase-3 expression in proliferating HPC03A/07 cells under DHA treatment

a, c, e, g depict the percentage of positive cells relative to absolute cell numbers; b, d, f, h show the percentage change of the marker in DHA treated cultures compared to EtOH treated cells. a) and b) Ki67 expressing cells c) and d) activated Caspase-3 expressing cells. Abbreviations: EtOH: Ethanol control; DHA: Docosahexaenoic acid Abbreviations: (p-values were generated using Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), **p<0.01, ***p<0.001).

3.1.4 Docosahexaenoic acid increases the proportion of dividing cells and neuroblasts while decreasing apoptosis in differentiating HPC03A/07 cells

To assess the effect of DHA during differentiation, HPC03A/07 cells were treated with DHA as described in Section 3.1. The percentage of Ki67 positive cells was significantly increased by 27.2±3.4%, $p<0.05$ compared to EtOH control treated cells to a total of 35.2±0.9%, (Figure 3-5a,b), so was the proportion of Dcx expressing cells by 13.5±0.7%, $p<0.05$ to a total of 18.5±0.1%, (Figure 3-5c,d). The percentage of activated Caspase-3 expressing cells on the other hand was significantly decreased by -27.8±2.3%, $p>0.001$ to a total of 9.9±0.5%, (Figure 3-5g,h). Whereas MAP2 was non significantly decreased (total of 15.2±1.6%, Figure 3-5e,f). SOX2 or Nestin expression showed no changes.

The increase of the proportion of dividing cells in HPC03A/07 cultures after 7 days of differentiation might be due to decreased apoptosis and therefore result in increased survival. DHA treatment further increases the percentage of early neurons (Dcx) but not mature neurons (MAP2), suggesting that DHA treatment increases differentiation into neuroblasts but the maturation to mature neurons might require more time. It would be interesting to see the changes after a longer e.g. 14 days differentiation period. Further double labelling BrdU incorporating cells for neuronal markers and cell death markers would shed light on whether all the changes can be explained through increased survival. Moreover double labelling activated Caspase-3 positive cells with neuronal markers to examine which cells die.

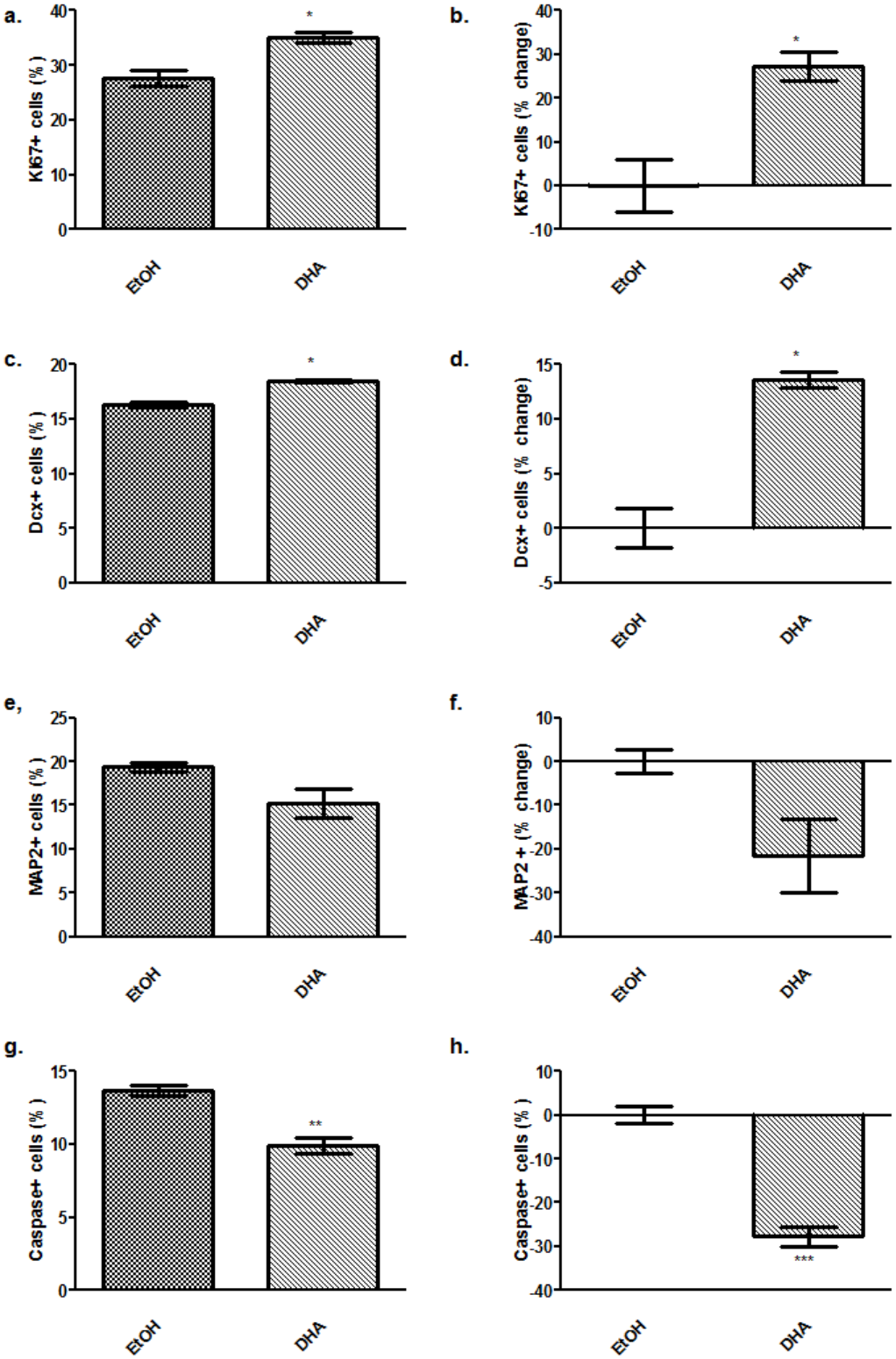


Figure 3-5 Ki67, Dcx, MAP2 and Caspase expression in differentiating HPC03A/07 cells under DHA treatment

a, c and e show the percentage of Ki67, DCX, MAP2 and activated Caspase-3 expressing cells respective in DHA treated and control cultures. b, d and f show the percentage change of each marker in EPA treated cultures compared to control EtOH treated cells. DHA treatment results in increased Ki67 (a, b), Dcx (c, d) expression and decreased activated Caspase-3 expression. No changes in MAP2 expression. Abbreviations: EtOH: Ethanol control; DHA: Docosahexaenoic acid (p-values were generated using Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001)

3.2 The stilbenoid Resveratrol increases the proportion of dividing cells and neuronal cells in HPC03A/07 cells

In order to determine the effects of the stilbenoid RSVL on proliferating and differentiating HPC03A/07 cells, cells were cultured as described in Section 2.1.1. After 24h under proliferating conditions RSVL treatment was started and for the proliferation assay cells were maintained for further 6 days under proliferation conditions before being fixed with 4% PFA. Media was changed after 3 days. For the differentiation assay cells were cultures for 24h before, RSVL was added and maintained for 6 days, media was changed after 3 days. Differentiation was started after a total of 6 days under proliferation conditions and cultures were maintained for 7 days under differentiation conditions before being fixed for immunocytochemistry. Cultures were then stained for the proliferation marker Ki67 and the apoptosis marker activated Caspase-3. Cultures were also double labelled for the NPC marker SOX2 and Nestin. In the differentiation experiment, cells were also stained for the neuroblast marker Dcx and the mature neuronal marker MAP2.

3.2.1 Titration of Resveratrol concentration

To determine the concentration of RSVL that promoted the most proliferation, HPC03A/07 cells were cultured for 3 days under proliferation conditions while being treated with various concentrations of RSVL: 10 µM, 5µM, 1µM, 500nM, 300nM and 100nM. Concentration range was established based on literature research (0.1µM-10µM (Bruedigam et al., 2011) and preliminary experiments (data not shown)). Cultures were then fixed with 4% PFA and immunocytochemically stained for the proliferation marker Ki67. At 10µM most of the cells died after 24h (data not shown). Figure 3-6 shows that at 5µM the

percentage of cells expressing Ki67 was non-significantly decreased, at 1µM the percentage of Ki67 expressing cells was increased by 40.4±8.4%, p<0.01. At lower RSVL concentrations than 1µM the percentage of Ki67 expressing cells was still increased but to a lesser extent (500nM: 31.1±6.9%, p<0.05, 300nM: 30.2±6.3%, p<0.01, 100nM 11.2±7.6%, p>0.05.). In conclusion, RSVL caused cell death at 10µM and a decrease in the percentage of dividing cells in HPC03A/07 cells at the 5µM, but significantly increased the percentage of dividing cells at lower concentration, with 1µM giving the greatest effect.

Based on my hypothesis that RSVL has a positive effect on proliferating and differentiating HPC03A/07 cells I chose 1µM as it increased the proportion of dividing cells most.

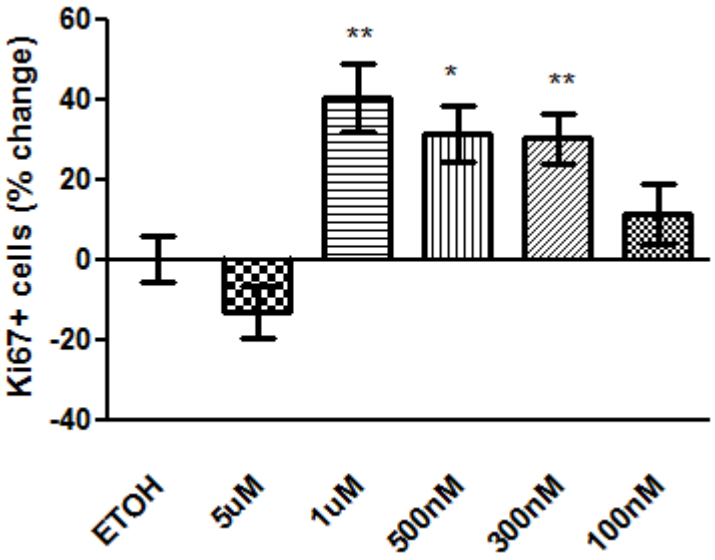


Figure 3-6 RSVL titration curve after 3 days of proliferation
Quantification of Ki67 positive cells in HPC03A/07 cultures treated with decreasing concentrations of RSVL. Abbreviations: EtOH: Ethanol, RSVL: Resveratrol; (biological replicates n=1, technical replicates n=3; P-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01)

3.2.2 Resveratrol increases the proportion of dividing cells in proliferating HPC03A/07 cells

In order to determine the effects of RSVL treatment on proliferation in HPC03A.07 cells, cultures were treated with 1µM RSVL as described in Section 3.2. RSVL treatment significantly increased the percentage of Ki67 positive cells by 21.4±2.9%, $p<0.001$ compared to EtOH treated control cultures to a total of 41.9±1.0% (Figure 3-7a,b). The proportion of activated Caspase-3 expressing cells was not significantly changed (total of 11.6±1.3%), Figure 3-7c,d. The percentage of SOX2 or Nestin expressing cells showed no changes.

This suggests that RSVL impacts on dividing cells in proliferating HPC03A/07 without affecting apoptosis, which suggests that this increase is due to an increased rate of division (proliferation) and not due to increased survival.

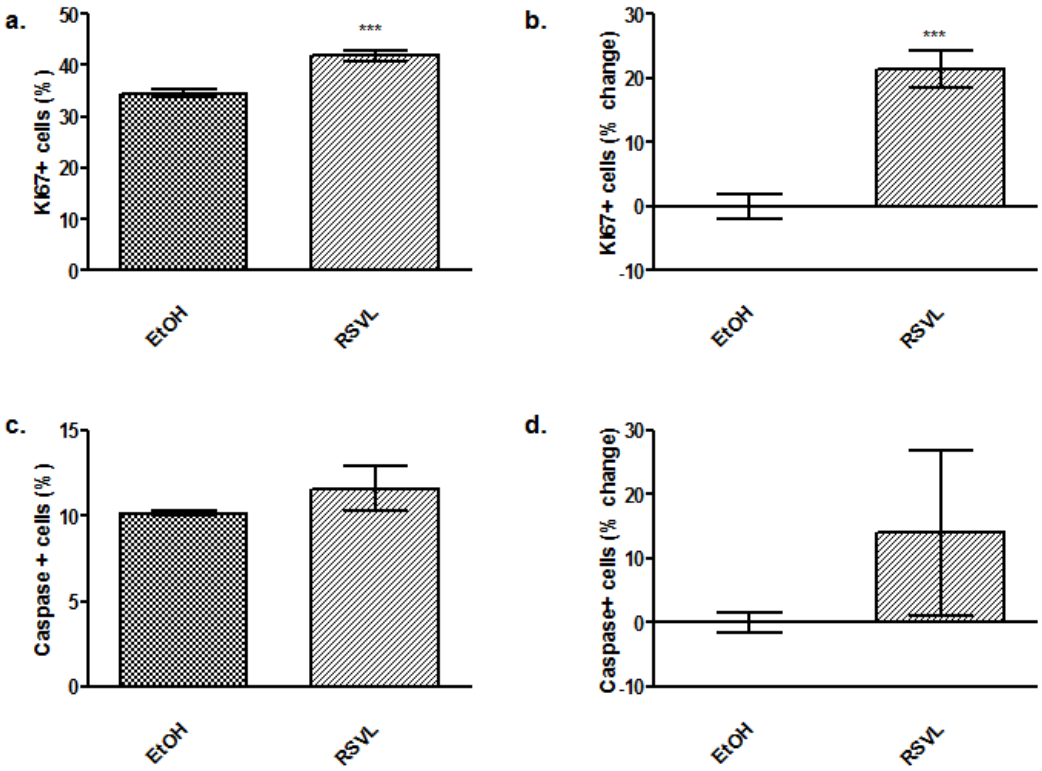


Figure 3-7 Ki67 and activated Caspase-3 expression in proliferating HPC03A/07 cells under RSVL treatment

a, c, e, g depict the percentage of positive cells relative to absolute cell numbers, b, d, f, h show the percentage change of the marker in RSVL treated cultures compared to EtOH treated cells. a) and b) Ki67 expressing cells c) and d) activated Caspase-3 expressing cells. Abbreviations: EtOH: Ethanol control; RSVL: Resveratrol (p-values were generated using Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), ***p<0.001)

3.2.3 Resveratrol increases the proportion of dividing cells, apoptosis and mature neurons

To assess the effects of RSVL on HPC03A/07 under differentiation conditions cultures were treated as described in Section 3.2. The percentage of Ki67 positive cells was significantly increased by 27.3±1.8%, $p<0.01$ compared to control treated cultures to a total of 35.2±0.5%, Figure 3-8a,b. Also MAP2 was significantly increased by 52.3±0.3%, $p<0.0001$ to a total of 36.7±0.1%, Figure 3-8e,f, as was the percentage of activated Caspase-3 expressing cells by 33.1±8.8%, $p<0.01$, to a total of 23.5±1.6%, Figure 3-8g,h. Whereas the percentage of Dcx expressing cells was not changed (total of 28.3±1.3%), (Figure 3-8c,d). SOX2 and Nestin expression showed no changes.

These results indicate that RSVL significantly increases apoptosis but also the proportion of dividing and differentiating cells in form of mature neurons (MAP2). This might suggests that RSVL treatment during differentiation pushes the cells into cell fate commitment and also division on a fast route that leads to cell death, indicating increased asymmetric division: cells divide with one daughter cell remaining a dividing progenitor cell, the other cells undergoing cell fate commitment into the neuronal lineage. The increase in mature neurons but not neuroblasts indicates that cells that have committed to the neuronal lineage have already matured at 7 days differentiation and/or that some cells have died during neuronal maturation or cell division, due to an increased cell cycle. To verify the suspected increased proliferation rate, BrdU staining will have to be conducted. Also double staining for apoptotic cells with marker for proliferation and differentiation will reveal the type of dying cells.

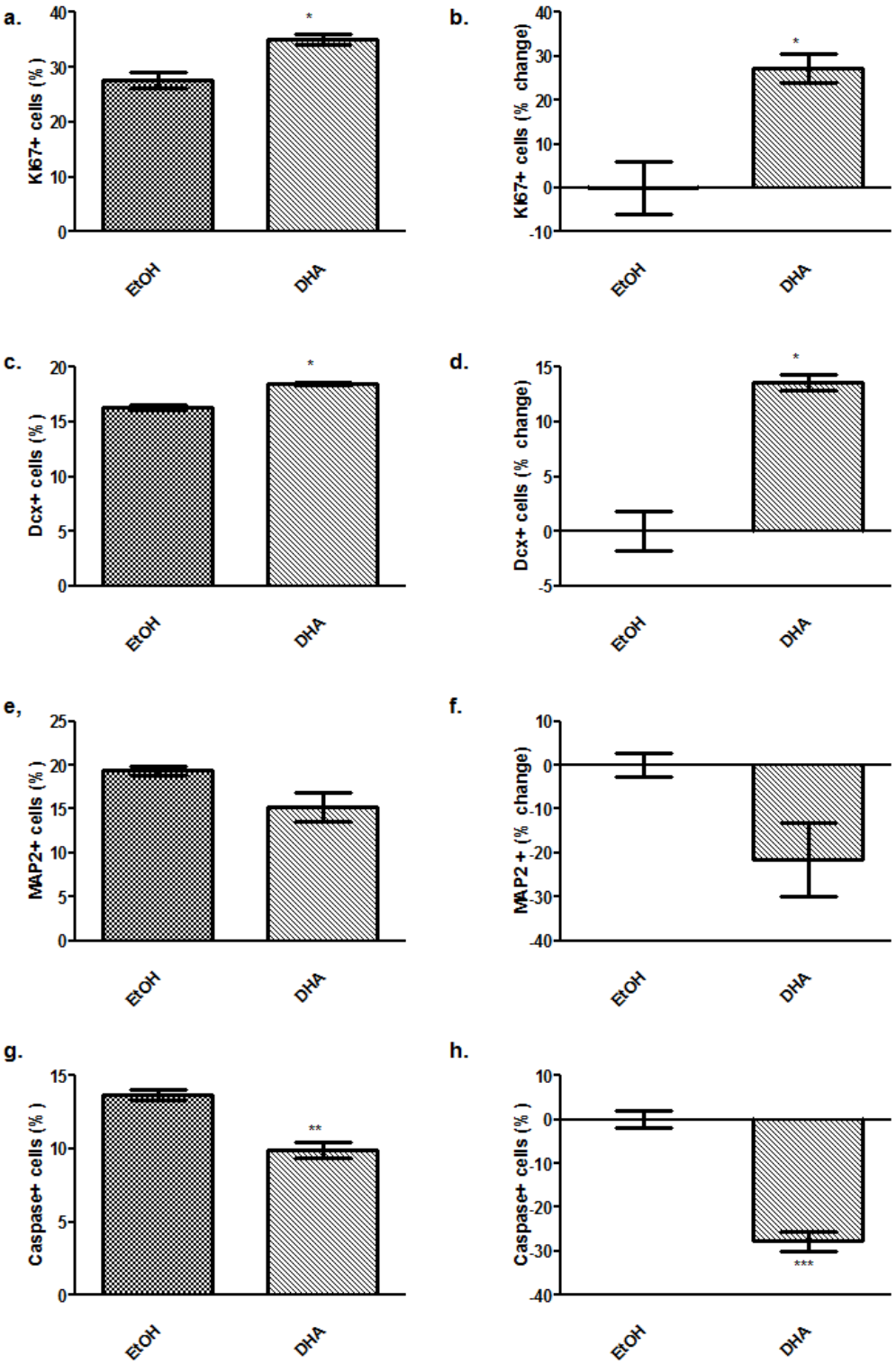


Figure 3-8 Ki67, Dcx, MAP2 and activated Caspase-3 expression in differentiating HPC03A/07 cells

a, c and e show the percentage of Ki67, DCX, MAP2 and activated Caspase-3 expressing cells respective in RSVL treated and control cultures. b, d and f show the percentage change of each marker in RSVL treated cultures compared to control EtOH treated cells. RSVL treatment results in a significant increase in Ki67 expressing cells (a,b) and MAP2 expressing cells (e,f), a signifcant decrease in activated Caspse-3 expressing cells, but no change in Dcx expressing cells (c,d). Abbreviations: EtOH: Ethanol control; RSVL: Resveratrol (p-values were generated using Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), **p<0.01, ***p<0.001, ****P<0.000)

3.3 Summary

Table 3-1 summarises the effect of the Omega-3s EPA and DHA and the stilbenoid RSVL on proliferating and differentiating HPC03A/07 cells described in Sections 3.1, 3.2 and 0. EPA and DHA both decrease apoptosis and increase the proportion of dividing cells and of neuronal cells, with EPA increasing the proportion of mature neurons and DHA increasing the proportion of neuroblasts. Further, EPA treatment during differentiation is the only condition observed where the expression of the NPC marker Nestin was significantly decreased, suggesting an increase in survival and differentiation. It might be possible that under EPA treatment at an earlier time point in differentiation (3 days) a significant increase of Dcx positive neuroblasts could occur and that after 7 days these neuroblasts have already matured into MAP2 positive neurons. Whereas cell differentiation into mature neurons under DHA treatment might take longer and the proportion of MAP2 positive cells might only increase after e.g. 14 days under differentiation conditions. On the other hand the survival of the neuroblasts or the mature neurons might not be maintained during DHA treatment, and the cells might die on their way to becoming mature neurons. It will be necessary to characterise the nature of the apoptotic cells to explore what cell types die, especially as the proportion of activated Caspase-3 positive cells in DHA treated cultures is almost 2% higher compared to EPA treated cells. RSVL increases the proportion of dividing cells, mature neuronal cells and apoptosis. This suggests that RSVL treatment increases the rate of cell fate commitment and asymmetric division, however to confirm this BrdU staining will be necessary. The proportion of SOX2 and Nestin expressing cells showed no or very minor changes throughout the experiments of this thesis, except during *Klotho* over-expression in 4.2. As described in Section 2.1.1, 7 days of

differentiation might not be long enough for SOX2 and Nestin to be degraded, leading to fate committed cells still expressing SOX2 and Nestin. Also changes during proliferation as seen here with the decrease in Ki67 positive cells might not be reflected in the SOX2 and Nestin population as this human cell line might require longer periods of differentiation to reflect changes in SOX2 and Nestin population. Further, an increase in Ki67 cells cannot be reflected in the SOX2 and Nestin population as almost 100% of cells are already expressing these markers. These observations were also made by colleagues within the department and appear to be typical for this cell line. Co-labelling with markers for dividing and differentiating cells as well as extending the differentiation period are necessary experiments to further characterise this cell line.

To validate the Cortisol Stress Model to enable the investigation of the potential stress preventing properties of EPA, DHA and RSVL, I next went on to examine the effects of stress-relevant Cortisol concentrations on HPC03A/07 cells.

EPA		
	3d pro	7d diff
Ki67	↑↑↑	↑↑
Dcx		-
MAP2		↑↑↑↑
Caspase	↓	↓↓
SOX2	-	-
Nestin	-	↓

DHA		
	3d pro	7d diff
Ki67	↑↑↑↑	↑
Dcx		↑
MAP2		-
Caspase	-	↓↓
SOX2	-	-
Nestin	-	-

RSVL		
	3d pro	7d diff
Ki67	↑↑↑	↑↑↑
Dcx		-
MAP2		↑↑↑↑
Caspase	-	↑↑
SOX2	-	-
Nestin	-	-

Table 3-1 Summary of the effect of EPA, DHA, RSVL on HPC03A/07 during proliferation and differentiation.
↑ indicates the level of significantly increased expression; ↓ the level of signnificanlty decreased expression; - for no significant changes. DHA: Docosahexaenoic acid, EPA: eicosapentaenoic acid; RSVL: Resveratrol; Pro: Proliferation; Diff: Differentiation

Effects of Cortisol on HPC03A/07 (Stress Model)

Glucocorticoid hormones, such as the human endogenous glucocorticoid Cortisol, are consistently elevated in severely depressed patients and in animal models of chronic stress and depression. Importantly, high levels of glucocorticoid hormones decrease adult hippocampal neurogenesis in rodents (Nestler et al., 2002). See 1.2.2 and 2.1.5.1. Anacker et al. showed that Cortisol only decreases proliferation and neurogenesis when cells are treated during the mitotic phase (Anacker et al., 2011), which is why I treated the cultures with Cortisol for 3 days under proliferation conditions before starting differentiation.

Here I will first confirm and validate the negative effects of high concentrations (100µM) of Cortisol on HPC03A/07 during proliferation and differentiation in order to be able to use this model of stress to investigate the potential of EPA, DHA and RSVL in preventing the effect of stress on hippocampal neurogenesis.

3.3.1 Cortisol decreases the proportion of cells dividing while increasing apoptosis

Cultures were treated as described previously in Figure 2-9 in Section 2.1.5.1. 100µM Cortisol were added after 4 days of proliferation and cells were maintained for a further 3 days under proliferation conditions. The percentage of Ki67 positive cells was significantly decreased by -23.97±7.6%, $p<0.05$ compared to the EtOH control cultures to a total of 30.0±2.1% (Figure 3-9a,b). The percentage of activated Caspase-3 expressing cells was significantly increased by 72.66±23.5%, $p<0.05$ to a total of 15.5±2.1% (Figure 3-9c,d). SOX2 or Nestin expression showed no changes, please see Section 3.3. These data suggest that Cortisol decreases the proportion of dividing cells and

increases apoptosis in proliferating HPC03A/07 cells, affecting survival and cell division.

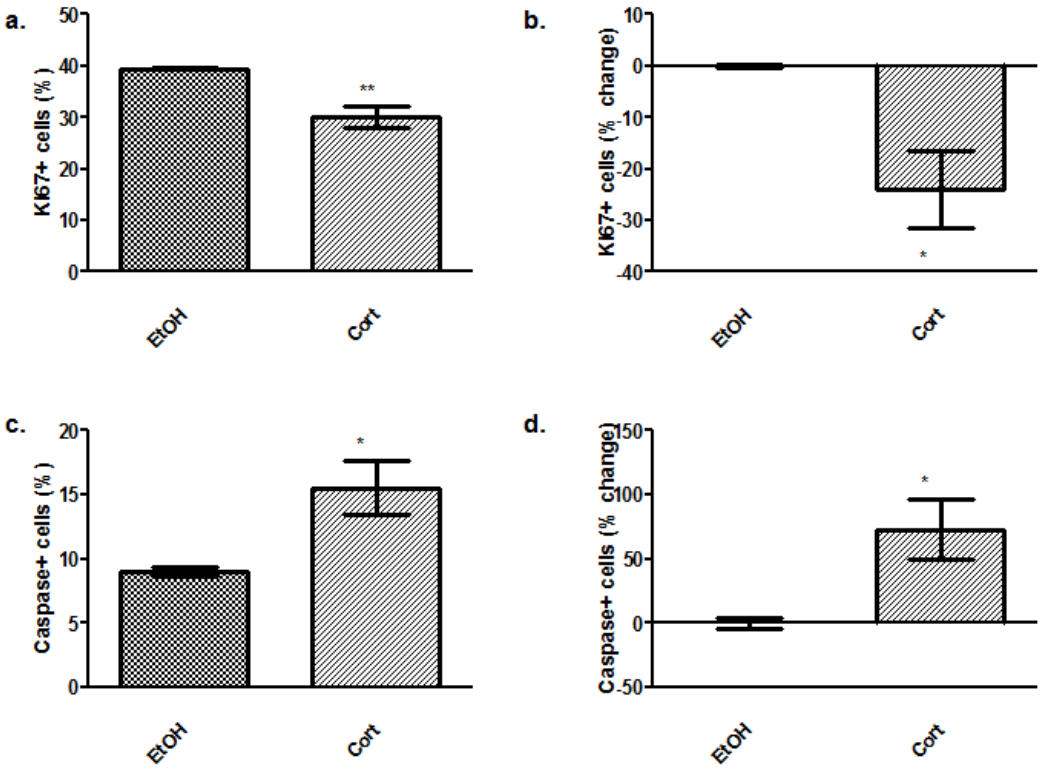


Figure 3-9 Ki67 and activated Caspse-3 expression in proliferating HPC03A/07 under Cortisol treatment

a, c, e, g depict the percentage of positive cells relative to absolute cell numbers, b, d, f, h show the percentage change of the marker in Cortisol treated cultures compared to EtOH treated cells. a) and b) Ki67 expressing cells c) and d) activated Caspase-3 expressing cells. Abbreviations: EtOH: Ethanol control; Cort: Cortisol, p-values were generated using Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01

3.3.2 Cortisol decreases the proportion of dividing cells and neuronal cells while increasing apoptosis in differentiating cells

To assess effects Cortisol has on differentiating HPC03A/07 cells, cultures were treated as described in Section 3.1. The percentage of Ki67 positive cells was significantly decreased by -17.53±4.5%, $p<0.05$ to a total of 24.8±0.7% (Figure 3-10a,b), as was the percentage of MAP2 expressing cells by -49.5±3.3%, $p<0.01$ to 9.8±0.6% (Figure 3-10e,f) but the percentage of activated Caspase-3 expressing cells was significantly increased by 43.1±5.9%, $p<0.01$ to a total of 19.6±1.1% (Figure 3-10g,h). On the other hand, there was no significant change in the percentage of Dcx (14.8±0.4%, Figure 3-10c,d), SOX2 or Nestin, please see Section 3.3.

These data confirm that Cortisol significantly decreases both the proportion of proliferating cells in the culture and the proportion of mature neurons. This could be explained by the increased cell death observed in these cultures.

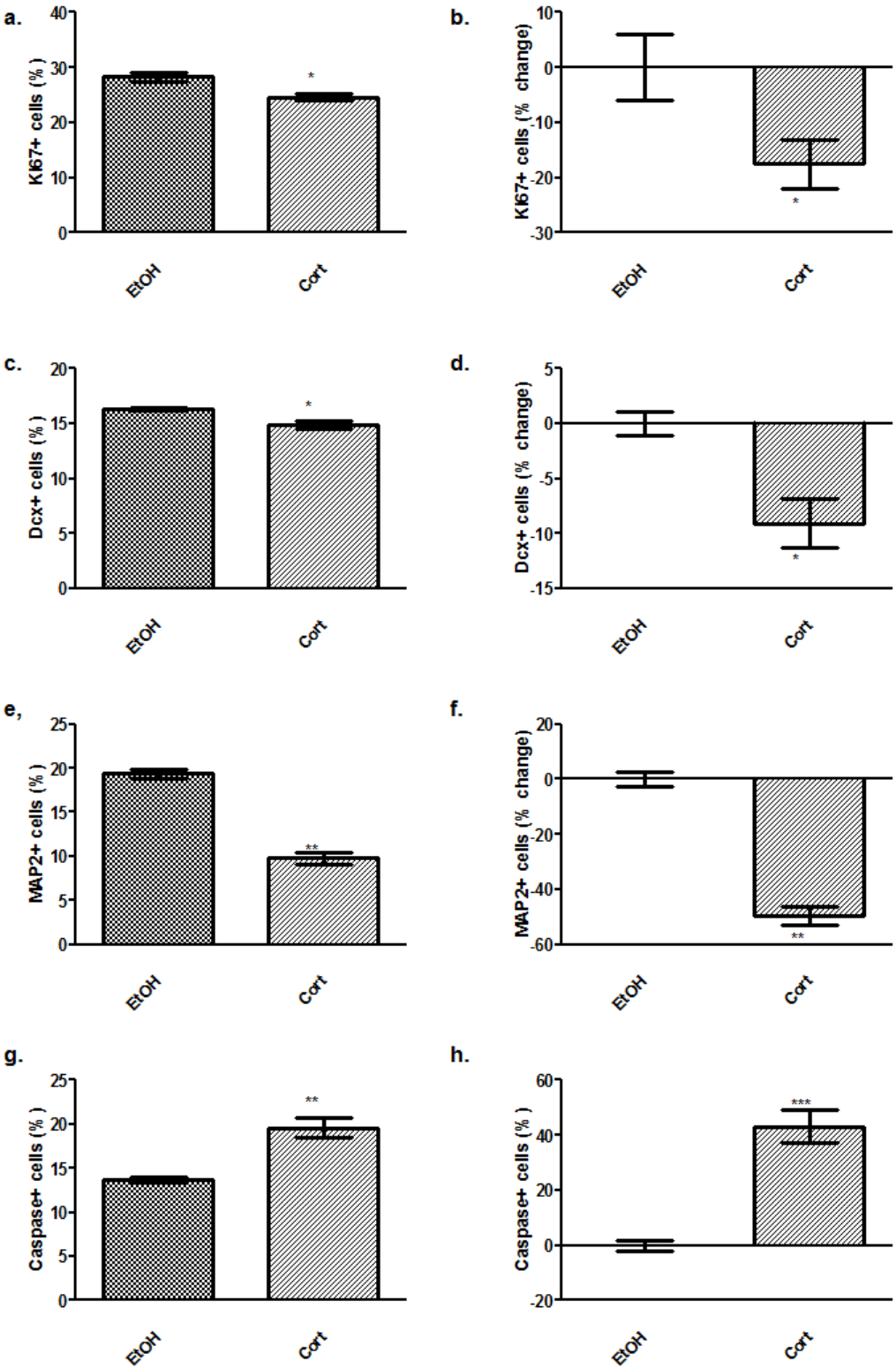


Figure 3-10 Ki67, Dcx, MAP2 and activated Caspase-3 expression in differentiating HPC03A/07 cells under Cortisol treatment

a, c and e show the percentage of Ki67, DCX and MAP2 expressing cells respective in Cortisol treated and control cultures. b, d and f show the percentage change of each marker in Cortisol treated cultures compared to control EtOH treated cells. Cortisol treatment results in a significant decrease in Ki67 expressing cells (a,b), Dcx expressing cells (c,d) and MAP2 expressing cells (e,f) and a significant increase in activated Caspse-3 expressing cells. Abbreviation: EtOH: Ethanol control; Cort: Cortisol, p-values were generated using Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001

3.4 Preventative effects of Omega 3 fatty acids EPA and DHA and the stilbenoid Resveratrol against stress relevant Cortisol levels in an *in vitro* stress model

The endogenous glucocorticoid Cortisol is consistently elevated in severely depressed patients and in animal models of chronic stress and depression (Pariante, 2009). Increased levels of glucocorticoid hormones over a long time also have detrimental effects on learning, memory and mood and decrease adult hippocampal neurogenesis in rodents (Mirescu and Gould, 2006; David et al., 2009). My results in Section 0 also showed that high concentrations of Cortisol (100µM) decrease proliferation and neurogenesis and increase apoptosis in HPC03A/07. Diet in form of Omega-3 fatty acids is known to have beneficial effects on learning, memory and mood (van Gelder et al., 2007; Fedorova et al., 2009). Interestingly, it has been hypothesised that anti-depressants may exert their effects by increasing AHN (Gould et al., 1997). This raised the question of whether certain diets may help protect against depression by increasing cell proliferation and neurogenesis in the brain and counteracting the negative effect of Cortisol. To test this hypothesis, in a controlled environment, I developed an *in vitro* stress model where a high concentration of Cortisol (100µM) was used to mimic the increased Cortisol levels and the decreased neurogenesis seen in depressed patients and in animal models of depressive behaviour. This model was used to investigate the potential preventative effects of the Omega-3s EPA and DHA and the stilbenoid RSVL by pre-treating HPC03A/07 cells with these compounds before starting the Cortisol treatment that would mimic stress.

In the following, I will describe the different treatment conditions: Step 1, 2 and 3 indicate a media change with renewed or changed treatment with Step 1 being pre-treatment with EPA, DHA (both 10µM) or RSVL (1µM), Step 2 start of Cortisol treatment and Step 3 start of differentiation. To determine the possible preventative properties of Omega-3s and RSVL on proliferation, differentiation and apoptosis using this *in vitro* stress model, HPCOA03/07A were pre-treated with EPA, DHA or RSVL for 3 days under proliferating conditions (Step 1) before treatment with 100µM Cortisol was started (Step 2). EPA, DHA or RSVL treatment was either continued together with Cortisol treatment for a further 3 days of proliferation to examine whether EPA, DHA or RSVL supplementation is needed during Cortisol treatment or stopped after the 3-day pre-treatment, to investigate whether pre-treatment only is sufficient to prevent the effects of Cortisol. After the treatment course cultures were fixed for immunocytochemistry (Section 2.2). For the differentiation assay, cultures were also pre-treated with EPA, DHA or RSVL (Step 1). Cultures were either only pre-treated (Step 1) before Cortisol treatment was started (Step 2) or continued for a further 3 days of proliferation together with the Cortisol, before differentiation was started (Step 3). Cultures were treated with Cortisol during the second 3 days of proliferation (Step 2) and the following 7 days of differentiation (Step 3) to assess the effects of Cortisol and EPA, DHA or RSVL on HPC03A/07 cells before they start differentiation. These conditions were based on the results of a colleague, Christoph Anacker, who used a similar model to show that presence of antidepressants during proliferation is essential to induce neuronal differentiation (Anacker et al., 2011). Cultures were treated with EPA, DHA or RSVL either during Step 1 only, during Step 1 and 2 or during

Step 1-3. After the differentiation period (Step 3) cultures were fixed with 4% PFA. See Figure 3-11.

Table 3-2 summarises the different experimental conditions during 1) proliferation and 2) differentiation, as well as giving the key to the abbreviations that will be used later in the graphs. **O** stands for treatment with Omega-3s EPA or DHA, **R** for Resveratrol, **C** for Cortisol and **–** stands for no treatment during this step with either EPA, DHA, RSVL or Cortisol. Briefly: During the proliferation assay (Table 3-2,1) the abbreviation **OO -C** indicates that cultures were pre-treated with EPA, DHA or RSVL (RR -C) for 3 days under proliferation conditions (Step1). After these 3 days, Cortisol treatment was started in parallel with the Omega-3 or RSVL treatment (Step 2) and maintained for 3 more days under proliferation conditions. **O- -C** indicates that after the 3 days pre-treatment, Cortisol treatment was started while Omega-3 or RSVL (R- -C) treatment was stopped, leading to pre-treatment only. **-C** indicates the negative control where cultures did not undergo any pre-treatment (Step 1) before Cortisol treatment was started after 3 days of proliferation and maintained for further 3 days of proliferation. **OO** indicates the positive control where cultures have been continuously treated with EPA, DHA or RSVL (RR) for 6 days under proliferation conditions and were not exposed to Cortisol.

For the differentiation assay explained in Table 3-2,2 **OOO -CC** indicates that cultures were pre-treated with EPA, DHA or RSVL (RRR -CC) for 3 days under proliferation conditions (Step 1). After these 3 days Cortisol treatment was started in parallel with the Omega-3 or RSVL treatment (Step 2) and maintained for 3 more days under proliferation conditions. Differentiation was started and Omega-3 or RSVL and Cortisol treatment was continued for 7 days under

differentiation conditions (Step 3). This condition mirrors full supplementation before and during stress under proliferation and differentiation conditions.

OO- -CC indicates that after the 3 days pre-treatment Cortisol treatment was started in parallel with Omega-3 or RSVL (RR- -CC) and maintained for 3 more days under proliferation conditions (Step 2). Before differentiation was started (Step 3) Omega-3 or RSVL treatment was stopped and only Cortisol treatment was continued during differentiation. This condition mirrors EPA, DHA and RSVL supplementation before stress and during stress under proliferation conditions, however stress continues during differentiation but not the supplementation.

O-- -CC indicates cultures that were only pre-treated with Omega-3 or RSVL (R-CC) for 3 days under proliferation conditions (Step 1). Thereafter Omega-3 or RSVL treatment was stopped and cultures were maintained for 3 days under proliferation conditions (Step 2) followed by 7 days of differentiation conditions (Step 3) under Cortisol treatment. This condition mirrors pre-treatment only with EPA, DHA or RSVL before stress starts and the stress takes place during the following proliferation (Step 2) and differentiation (Step 3). **–CC** provides the negative control where cultures did not undergo any pre-treatment (Step 1) before Cortisol treatment was started after 3 days of proliferation and maintained for further 3 days of proliferation (Step 2) followed by 7 days under differentiation conditions (Step 3). **OOO** serves as positive control and indicates cultures that have been continuously treated with EPA, DHA or RSVL (RRR) during Step 1-3, and were not exposed to Cortisol. **EtOH** indicates vehicle control cultures that were treated with the equivalent concentration of Ethanol (1%) throughout the experiment.

After the course of each experiment cultures were fixed with 4% PFA and immunocytochemically stained. To assess the proportion of dividing cells and NPC, cultures were stained for the proliferation marker Ki67, the NPC markers SOX2 and Nestin. To examine the level of cell death, cultures were stained for activated Caspase-3. To assess neuronal differentiation, Dcx for early neurons and MAP2 for mature neurons were used.

In this Section, I will describe the potential preventative effects of each treatment.

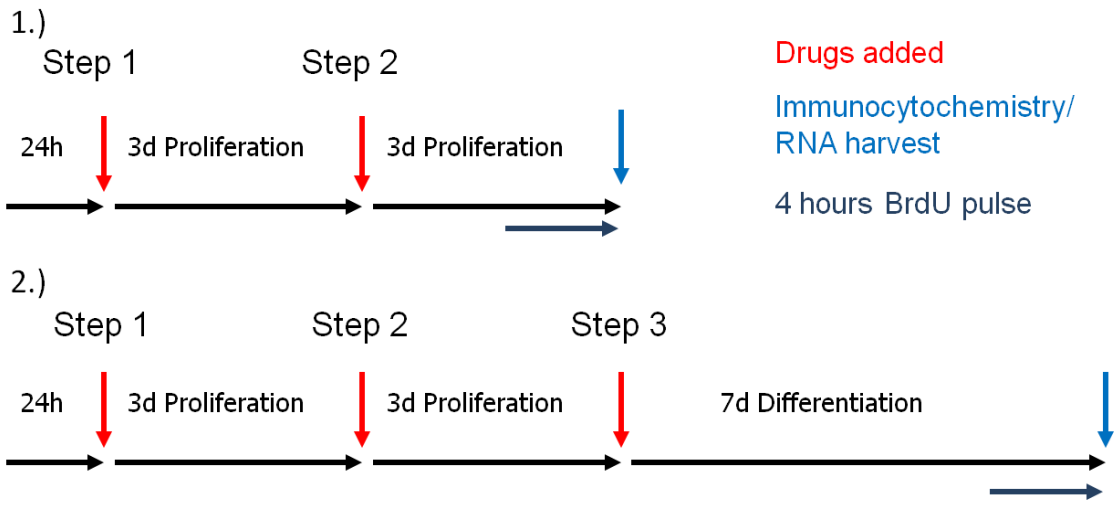


Figure 3-11 Timeline for Omega 3 prevention experiment using the Cortisol stress model on HPC0A07/03A cells.
1.) Proliferation assay, 2.) Differentiation assay Step 1: Cells were pre-treated with EPA, DHA or RSVL for 3 days under proliferation conditions. Step 2: Cortisol was added and EPA, DHA or RSVL treatment was either stopped or continued. For the proliferation assay cells were fixed for immunocytochemistry after an additional 3 days proliferation. Step 3: For the differentiation assay differentiation was started now and EPA, DHA or RSVL treatment was either stopped or continued leading to different combinations as described in Table 2-2 and Table 2-3

1.) Proliferation assay

	Step 1	Step 2
OO –C/ RR -C		
	3d pro	3d pro
	O/R	O/R
	-	Cort
O- -C/ R- -C		
	3d pro	3d pro
	O/R	-
	-	Cort
- C		
	3d pro	3d pro
	-	Cort
OO/ RR		
	3d pro	3d pro
	O/R	O/R
EtOH		
1% EtOH		

2.) Differentiation assay

Step 1	Step2	Step 3
OOO –CC/ RRR -CC		
3d pro	3d pro	7d diff
O/R	O/R	O/R
-	Cort	Cort
OO- -CC/ RR- -CC		
3d pro	3d pro	7d diff
O/R	O/R	-
-	Cort	Cort
O- CC/ R- CC		
3d pro	3d pro	7d diff
O/R	-	-
-	Cort	Cort

Step 1	Step 2	Step 3
- CC		
3d pro	3d pro	7d diff
-	Cort	Cort
OOO/ RRR		
3d pro	3d pro	7d diff
O/R	O/R	O/R
EtOH		
1% EtOH		

Table 3-2 Conditions for the omega-3 prevention experiment using the cortisol stress model

under 1.) proliferating and 2.) differentiation conditions. Step 1: Cells were pre-treated with EPA, DHA or RSVL for 3 days of proliferation, Step 2: Cortisol was added, Step 3: Differentiation was started. O: DHA/ EPA; R: RSVL; C/ Cort: Cortisol; -: no treatment; Pro: Proliferation; Diff: Differentiation

3.4.1 Preventative effects of EPA on proliferating and differentiating HPC03A/07 cells in a Cortisol Stress Model

In order to assess the effect of EPA pre-treatment on Cortisol-stressed HPC03A/07, cells were treated with 10µM EPA and 100µM Cortisol as described in Section 3.4 and immunocytochemical analysed to investigate changes in proliferation, differentiation and apoptosis. In Section 0 I showed that Cortisol decreases the percentage of dividing and differentiating cells while increasing apoptotic cells over control treatment, proving that the Cortisol Stress Model used in the following experiments works. I have also shown that EPA alone increases the percentage of dividing and neuronal cells but decreases apoptotic cells. Now I am investigating whether EPA has the potential to prevent the effects of Cortisol

3.4.1.1 EPA pre-treatment prevents the Cortisol-induced decrease of the proportion of dividing cells and increase of apoptosis in proliferating HPC03A/07

To investigate the preventative effects of EPA in the Cortisol stress model in proliferating HPC03A/07, cultures were treated with EPA and Cortisol as explained in Section 3.4 / Table 3-2,1. In the **OO –C** group, where cultures were supplemented with EPA before and during stress the percentage of Ki67 positive cells is significantly increased by 28.5±3.2%, $p<0.001$ compared to the Cortisol negative control (-C) to a total of 39.8±0.9% (Figure 3-12a,b). The percentage of apoptotic cells was significantly decreased by -32.9±3.5%, $p<0.05$ to a total of 8.8±0.5%. Also in the **O- -C** group cultures that were pre-treated only before Cortisol was added, the percentage of dividing cells was significantly increased by 26.5±2.0%, $p<0.001$ to 39.2±0.6% whereas the percentage of apoptotic cells was decreased by -31.6±3.8% to 6.9±1.9%. SOX2

and Nestin showed no changes; please see Section 3.3 for a possible explanation.

Both treatment groups, continuous supplementation (**OO –C**) and pre-treatment only (**O- -C**) could prevent the effects of Cortisol, namely the decrease of dividing cells and increase of apoptosis. Both treatments increased the proportion of dividing cells to the level of EPA only (**OO**) (39.3±0.6%) and above the **EtOH** control group (34.5±0.9%). None of the treatments were able to decrease the percentage of apoptotic cells to the level of EPA only treatment (**OO**) (5.4±1.5%) but to the level of the **EtOH** control (9.0±0.5%). This indicates an increase in survival in HPC03A/07 cells after EPA treatment in the Cortisol stress model.

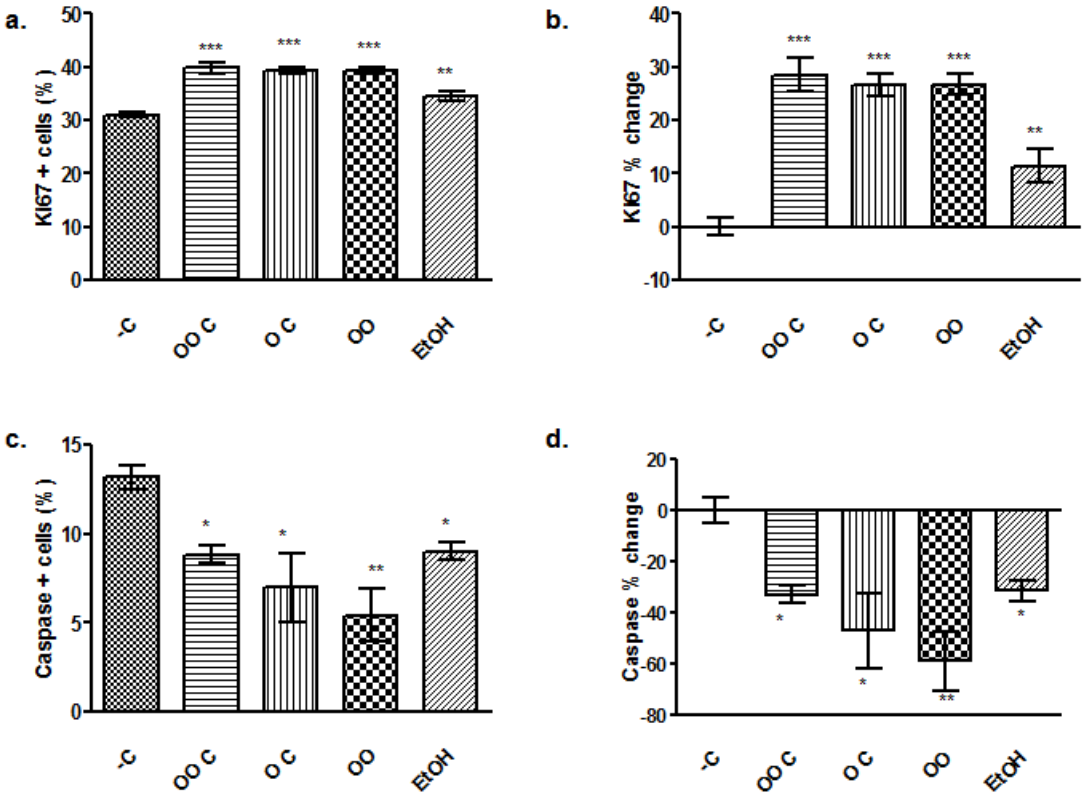


Figure 3-12 Ki67 and activated Caspase-3 expression In HPC03A/07 cells treated with EPA under the Cortisol Stress Model

a, c, e, g depict the percentage of positive cells relative to absolute cell numbers; b, d, f, h show the percentage change of the marker in treated cultures compared to Cortisol treated cells. a) and b) Ki67 expressing cells c) and d) activated Caspase-3 expressing cells; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), ***p<0.001; See Table 3-2 for abbreviations.

3.4.1.2 EPA pre-treatment prevents the Cortisol-induced decreases of dividing and neuronal cells as well as increase of apoptosis in differentiating HPC03A/07

To investigate the preventative effects of EPA in the Cortisol stress model in differentiating HPC03A/07, cultures were treated with EPA and Cortisol stress model as described in Section 3.4 / Table 3-2. In the **OOO –CC** group cultures were continuously supplemented with EPA before and during Cortisol treatment. The proportion of dividing cells was significantly increased by 31.0±6.5%, $p<0.001$ to a total of 29.8±0.4% (Figure 3-13a,b,d) compared to the Cortisol only group (-CC), as was the percentage of MAP2 positive cells by 154.7±8.3%, $p<0.001$ to total of 25.0±0.8% (Figure 3-15a,b,d). In turn, the percentage of activated Caspase-3 expressing cells was significantly decreased compared to the Cortisol group by -52.3±2.3%, $p<0.001$ to 12.2±0.6% (Figure 3-16a,b,d). Dcx (Figure 3-14a,b,d), SOX2 and Nestin were not changed compared to the Cortisol group. This suggests that this condition prevents the effects of Cortisol and moreover increase the percentage of dividing cells above the level of the EtOH control (total: 27.6±1.4%, Figure 3-13a) and close to the level of EPA only treatment (OOO) with a total of 33.6±0.5% (Figure 3-13a). Also the percentage of mature neuronal cells was increased above the EtOH group (19.4±0.5%, Figure 3-15a) and just below the EPA only group (OOO) with a total of 28.2±0.2% (Figure 3-15a). This treatment decreased the percentage of apoptotic cells below the EtOH control (14.21±0.7%, Figure 3-16a) but not quite to the level of EPA only (OOO) with 7.9±2.2% (Figure 3-16a). SOX2 or Nestin were unchanged. The absence of an increase in the percentage of Dcx expressing cells mirrors the results in Section 3.1.2 where EPA only treatment during differentiation increased the proportion of mature neurons but not the

proportion of neuroblasts. This suggests that this treatment (**OOO –CC**) prevents the effects of Cortisol and increases the proportion dividing cells and mature neurons by increasing cell fate commitment and maturation even above the level of the vehicle control. This might be due to survival as it also decreases the percentage of apoptotic cells. In the (**OO- -CC**) cultures, pretreated with EPA and treated together with Cortisol during proliferation but not during differentiation, the percentage of Ki67 cells was significantly increased by 31.3±4.6%, $p<0.001$ compared to the Cortisol control group (-CC) to a total of 33.3±1.9% (Figure 3-13a,b,e), so was the percentage of Dcx (by 26.5±3.3%, $p<0.05$, to a total of 16.0±0.4%, Figure 3-14a,b,e) and MAP2 positive cells by 155.4±2.7%, $p<0.001$ to 25.1±0.3% (Figure 3-15a,b,e). The percentage of activated Caspase-3 positive cells was in turn decreased by -44.4±2.3%, $p<0.001$ to 14.1±0.6% (Figure 3-16a,b,e). SOX2 or Nestin were unchanged, please see Section 3.3. This treatment (**OO- -CC**) increased the percentage of dividing cells, neuroblasts and mature neuronal cells above the level of vehicle control (Ki67: 27.6±1.4, Figure 3-13a; Dcx: 15.1±0.9%, Figure 3-14a; MAP2: 19.4±0.5%, Figure 3-15a) while decreasing the percentage of apoptotic cells to the level of the EtOH group with 14.2±0.7%, Figure 3-16a. These effects are mostly due to increased survival. In the **O-- -CC** group cultures were only pretreated with EPA before Cortisol treatment was started. The percentage of dividing cells was significantly increased by 24.1±2.0%, $p<0.001$ compared to the Cortisol only group to a total of 31.2±1.0% (Figure 3-13a,b,f), as was the percentage of neuroblasts with 29.4±6.7%, $p<0.05$ to 16.4±0.8% (Figure 3-14a,b,f) and the percentage of mature neuronal cells with 141.0±9.0%, $p<0.001$ to 23.6±0.9% (Figure 3-15a,b,f). The percentage of apoptotic cells was decreased by 52.2±5.6%, $p<0.001$ to 12.1±1.4% (Figure

3-16a,b,f). SOX2 or Nestin were unchanged. The pre-treatment only also is enough to prevent the effects of Cortisol and increased the percentage of dividing cells, neuroblasts and mature neurons above the level of the EtOH (Ki67: 27.6±1.4, Figure 3-13a; Dcx: 15.1±0.9% Figure 3-14a; MAP2: 19.4±0.5%, Figure 3-15a) and also decreases the percentage of apoptotic cells below the vehicle control (14.2±0.7%, Figure 3-16a). This suggests that even only pre-treatment with EPA can prevent the effects of Cortisol, namely decreasing the proportion of dividing and differentiating cells while increasing apoptotic cells over control treatment. This is most likely due to an increase in survival.

All three treatments prevent the negative effects of Cortisol by increasing the proportion of dividing and neuronal cells while decreasing apoptosis. The differences between the three treatment groups are only marginal, apart from the absent increase of Dcx in the OOO –CC group. Also during the EPA only treatment in Section 3.1.2, EPA only increased the proportion of mature neurons but not of neuroblasts, which might be due to EPA increasing the rate of cell cycle commitment as well as maturation of neuronal cells to an extent that most Dcx positive cells have already matured. However there is no difference in MAP2 positive cells in the three treatments, suggesting that in the OOO –CC group, neuronal differentiation might stop after 7 days with no more neuroblasts following or Dcx positive cells might have died. To investigate this matter further, different time points for differentiation (earlier and later) will be required. Also BrdU staining is required for investigating the proliferation rate which will allow answers regarding a depleted stem cell pool and examining the effects of EPA and Cortisol treatment during proliferation and differentiation will enable to draw further conclusions.

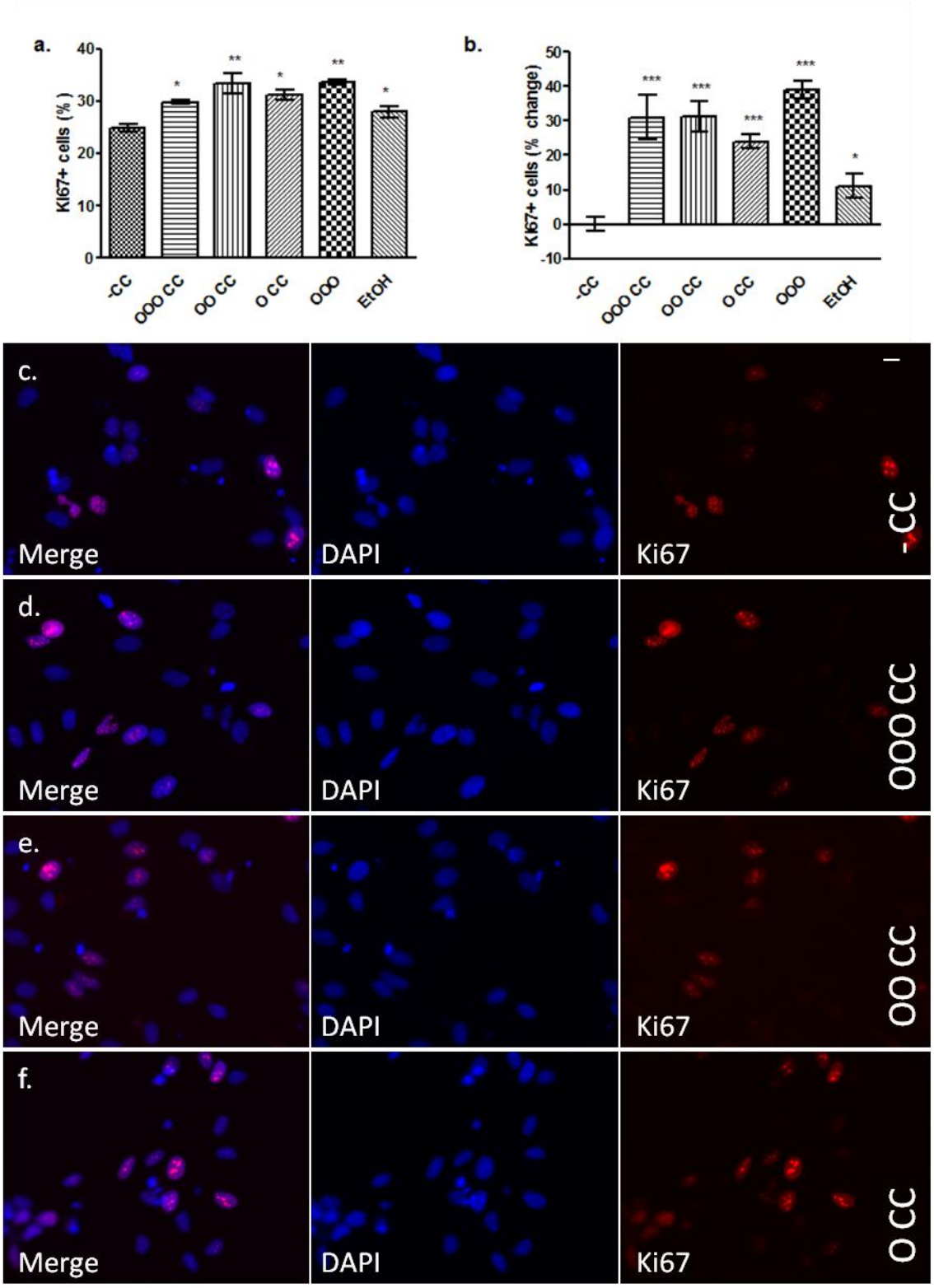


Figure 3-13 Ki67 expression in differentiating HPC03A/07 cells treated with EPA in the Cortisol stress model

a) Comparison of the absolute percentage of Ki67 positive cells. b) Percentage change of Ki67 expression c) Cortisol only; -CC d) continuous EPA treatment before and during Cortisol treatment; OOO –CC e) EPA pre-treatment and treated together with Cortisol during proliferation but not during differentiation, OO- -CC f) EPA pretreatment followed by Cortisol treament. (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

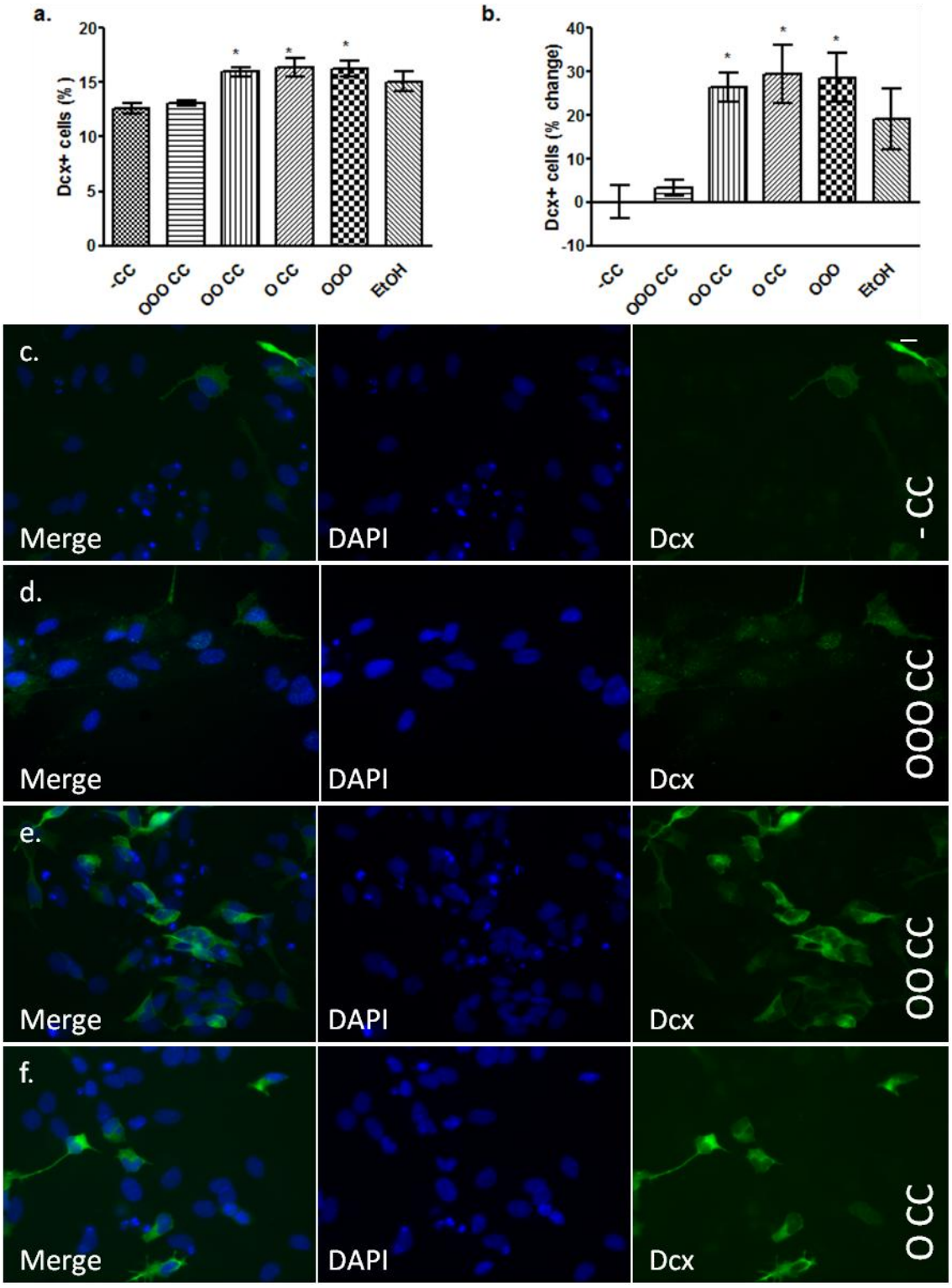


Figure 3-14 Dcx expression in differentiating HPC03A/07 cells treated with EPA in the Cortisol stress model

a) Comparison of the absolute percentage of Dcx positive cells. b) Percentage change of Dcx expression c) Cortisol only; -CC d) continuous EPA treatment before and during Cortisol treatment; OOO –CC e) EPA pre-treatment and treated together with Cortisol during proliferation but not during differentiation, OO- -CC f) EPA pretreatment followed by Cortisol treament (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

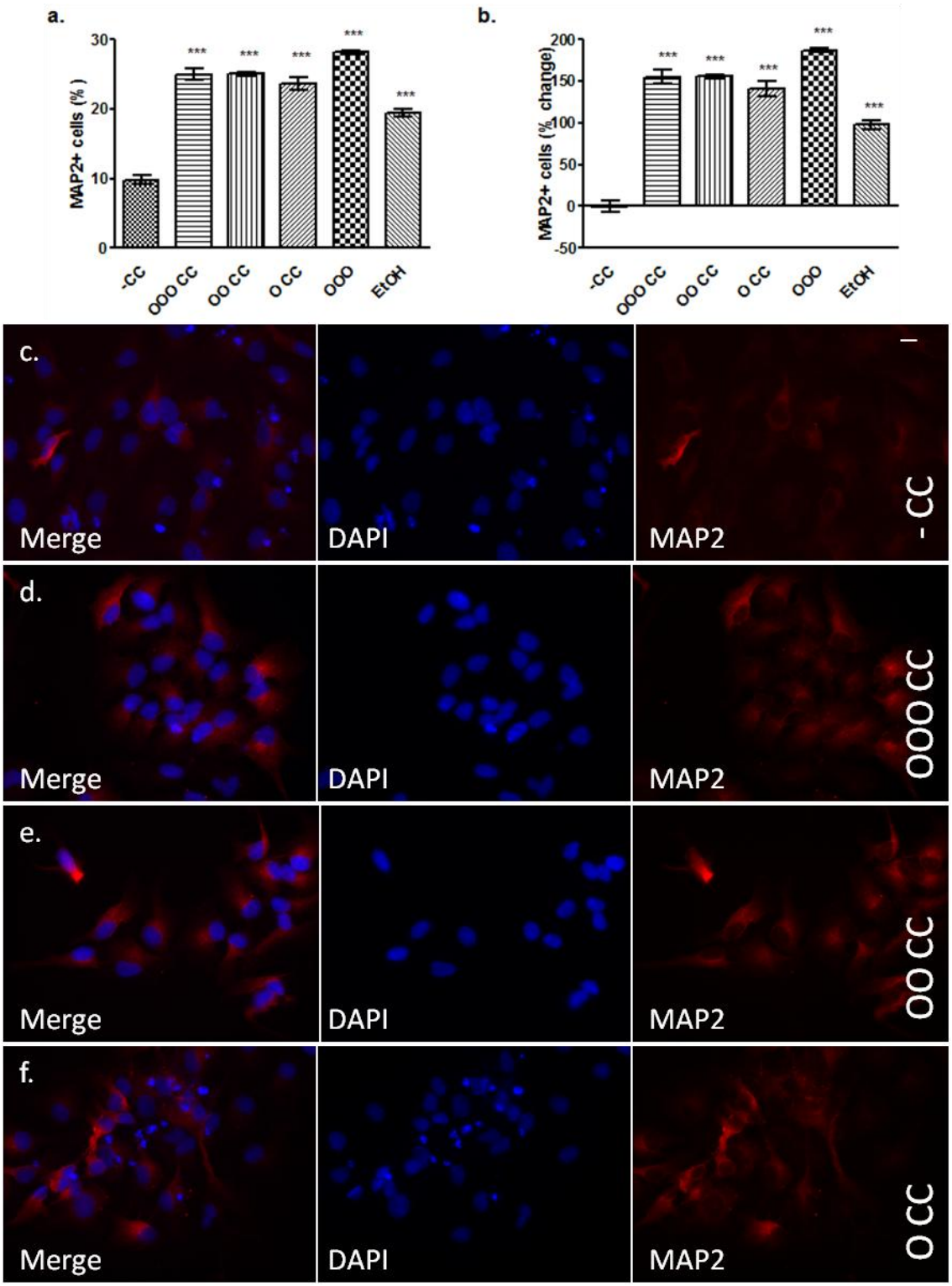


Figure 3-15 MPA2 expression in differentiating HPC03A/07 cells treated with EPA in the Cortisol stress model

a) Comparison of the absolute percentage of MAP2 positive cells. b) Percentage change of MAP2 expression c) Cortisol only; -CC d) continuous EPA treatment before and during Cortisol treatment; OOO –CC e) EPA pre-treatment and treated together with Cortisol during proliferation but not during differentiation, OO- -CC f) EPA pretreatment followed by Cortisol treament (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

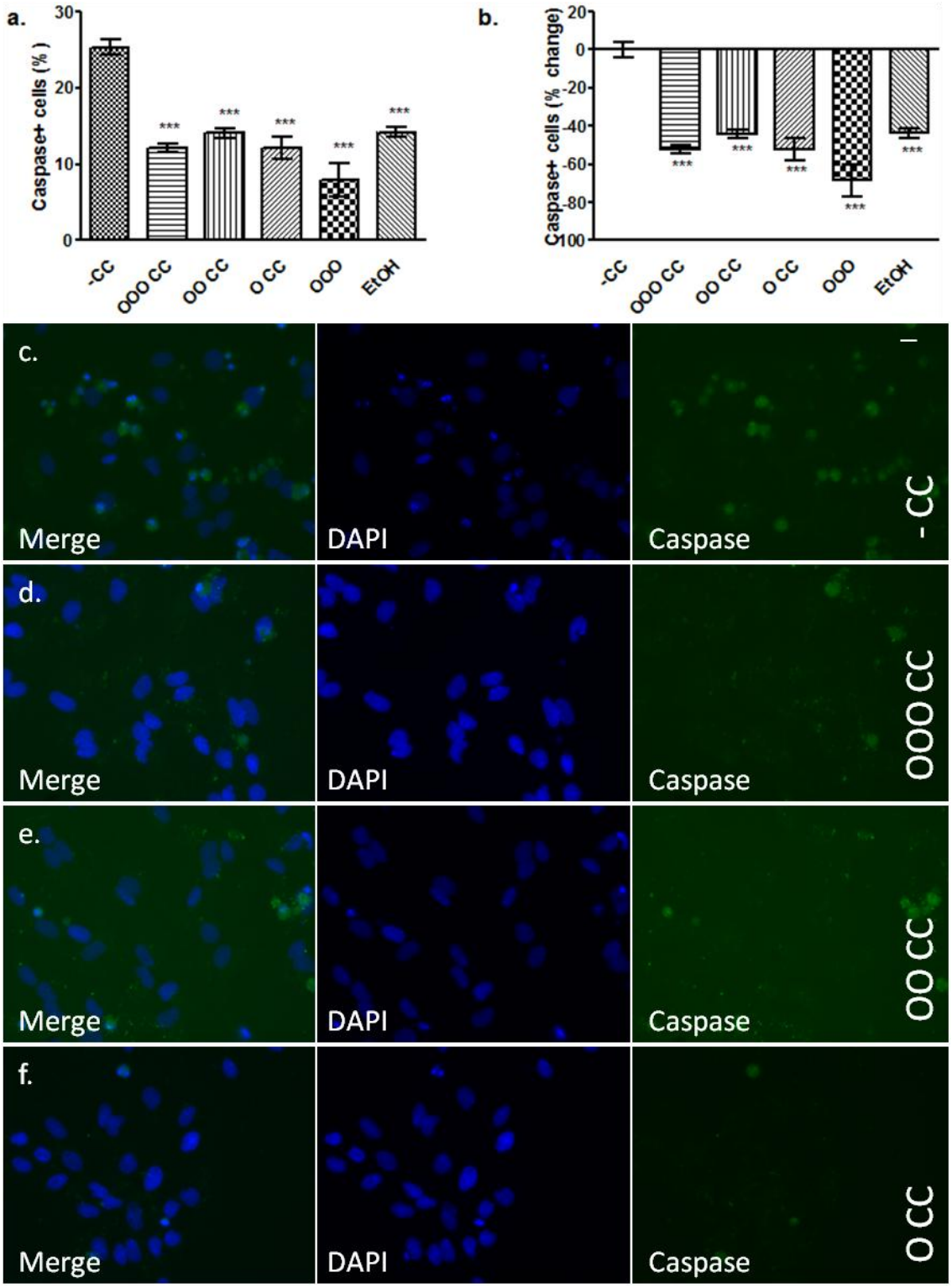


Figure 3-16 Activated Caspase-3 expression in differentiating HPC03A/07 cells treated with EPA in the Cortisol stress model

a) Comparison of the absolute percentage of activated Caspase-3 positive cells. b) Percentage change of activated Caspase-3 expression c) Cortisol only; -CC d) continuous EPA treatment before and during Cortisol treatment; OOO –CC e) EPA pre-treatment and treated together with Cortisol during proliferation but not during differentiation, OO- -CC f) EPA pretreatment followed by Cortisol treament (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

3.4.2 Preventative effects of DHA on proliferating and differentiating HPC03A/07 cells in a Cortisol Stress Model

In order to assess the effect of DHA pre-treatment on Cortisol-stressed HPC03A/07 cells, cultures were treated with 10µM DHA and 100µM Cortisol as described in Section 3.4 and immunocytochemically analyzed to investigate changes in proliferation, differentiation and apoptosis. Cortisol decreases the proportion of dividing and differentiating cells while increasing apoptotic cells over control treatment, as shown in Section 0. I have also shown that DHA alone increases the proportion of dividing and neuroblasts but decreases apoptotic cells (Section 3.1.3 and 3.1.4). In the following I am investigating whether DHA has the potential to prevent the effects of Cortisol

3.4.2.1 DHA pre-treatment prevents the Cortisol-induced decrease of dividing cells and increase of apoptosis in proliferating HPC03A/07

To investigate the preventative effects of DHA in the Cortisol stress model in proliferating HPC03A/07, cultures were treated with DHA and Cortisol as explained in Section 3.4 / Table 3-2,1. In the **OO –C** group cells were pretreated with DHA for 3 days and then treated with DHA and Cortisol together for further 3 days of proliferation. The percentage of Ki67 expressing cells was significantly increased by 17.9±2.1%, $p<0.05$ compared to the Cortisol negative control (-CC) to a total of 35.4±0.6% while the percentage of activated Caspase-3 positive cells was significantly decreased by -46.9±7.1%, $p<0.01$ to a total of 8.2±1.1%. SOX2 and Nestin were unchanged. This suggests that the increase in dividing cells was due to a big increase in survival. In the **O- -C** the percentage of Ki67 positive cells was increased by 25.3±1.5%, $p<0.05$ to a total of 37.5±0.4%, while also the percentage of apoptotic cells was decreased by

42.1±2.5%, $p<0.01$ to a total of 10.4±0.2%. SOX2 and Nestin did not change, please see Section 3.3 for a possible explanation.

Both treatment groups, the pre-treatment only and the continuous DHA treatment, could prevent and counter act the effects of the stress-mimicking Cortisol treatment. However, they did not reach the level of DHA only (OO) treatment (51.3±2.9%) and did not exceed the level of the vehicle control (EtOH, 39.5±0.1). On the other hand both decreased the percentage of apoptotic cells to the level of DHA only group (8.2±0.6%) and the EtOH control group (8.9±0.4%). This suggests that DHA pre-treatment prevents the negative effects Cortisol has on the proportion of dividing cells and apoptosis by increasing the percentage of dividing cells through an increase in survival.

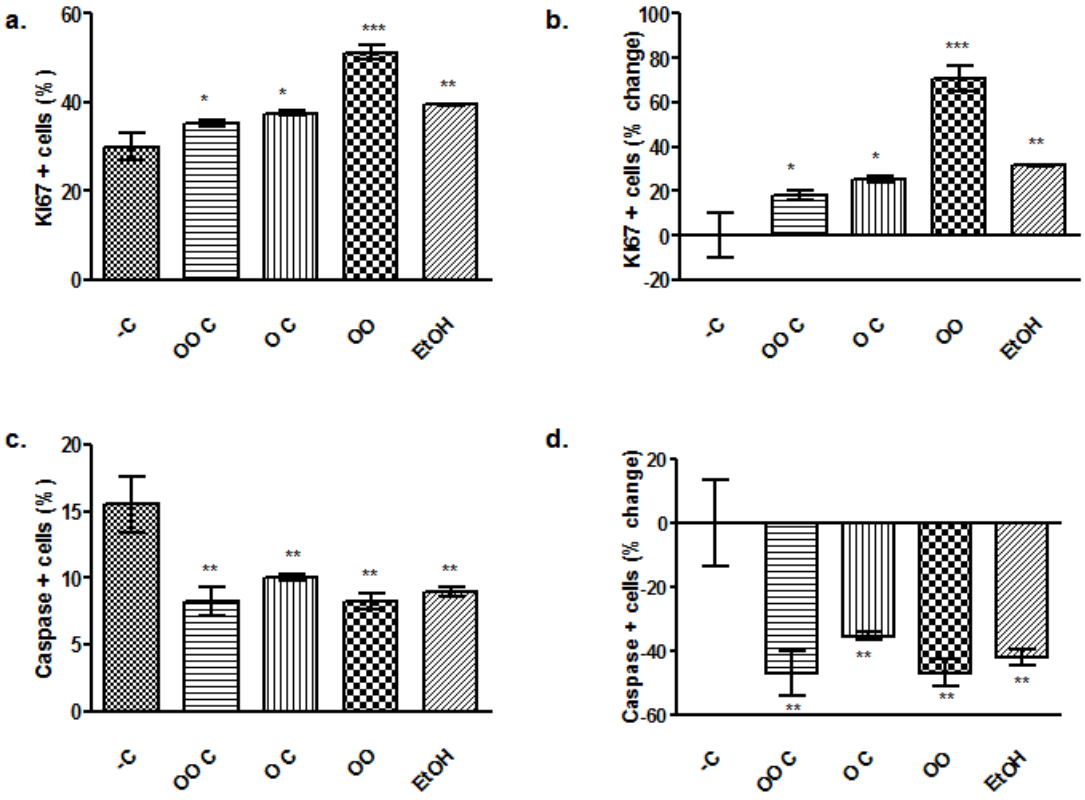


Figure 3-17 Ki67 and activated Caspase-3 expression In HPC03A/07 cells treated with DHA under the Cortisol Stress Model

a, c, e, g depict the percentage of positive cells relative to absolute cell numbers, b, d, f, h show the percentage change of the marker in Cortisol treated cultures compared to EtOH treated cells. a) and b) Ki67 expressing cells c) and d) activated Caspase-3 expressing cells; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations

3.4.2.2 DHA pre-treatment prevents the Cortisol-induced decrease of the proportion of dividing and neuronal cells as well as the increase of apoptosis in differentiating HPC03A/07

To investigate the preventative effects of DHA in the Cortisol stress model in differentiating HPC03A/07, cultures were treated with DHA and Cortisol as described in Section 3.4 / Table 3-2,2). In the **OOO –CC** group cultures were continuously supplemented with DHA before and during Cortisol treatment. The percentage of dividing cells was significantly increased by 42.0±1.6%, $p<0.001$ compared to the Cortisol only treated group (-CC) to a total of 35.3±0.7% (Figure 3-18a,b,d), also the percentage of Dcx (47.0±2.7%, $p<0.001$, total 21.8%, Figure 3-19a,b,d) and MAP2 positive cells were significantly increased (71.5±3.8%, $p<0.001$, total 20.2%, (Figure 3-20a,b,d). The percentage of activated Caspase-3 (Figure 3-21a,b,d), SOX2 or Nestin expressing cells was unchanged compared to the Cortisol group, please see Section 3.3. This suggests that this treatment prevents the effects of Cortisol by increasing the percentage of dividing cells, early and mature neuronal cells above the level of both DHA only (Ki67 35.2±0.9% Figure 3-18a; Dcx 18.5±0.1%, Figure 3-19a; MAP2 15.2±1.6%, Figure 3-20a) and EtOH control (Ki67 27.6±1.4, Figure 3-18a; Dcx 16.3±0.3%, Figure 3-19a; MAP2 19.4±0.5%, Figure 3-20a). This is not based on increased survival as DHA failed to prevent the apoptotic effects of Cortisol, which suggests that the decreasing effects of DHA on apoptosis during proliferation (Section 3.4.2.1) disappears when cultures were treated during differentiation.

The (**OO- -CC**) cultures were pretreated with DHA and treated together with Cortisol during proliferation but not during differentiation. The percentage of Ki67 cells in this assay was significantly increased to a total of 32.2±1.7%,

29.6±4.2%, p<0.001 higher compared to the Cortisol group (Figure 3-18a,b,e), as was the percentage of the neuronal marker Dcx (17.0±1.5%, p<0.05, to a total of 16.8±0.4%, Figure 3-19a,b,e) and MAP2 (26.1±3.5%, p<0.05 to a total 16.8%, Figure 3-20a,b,e). The percentage of activated Caspase-3 positive cells was significantly decreased by -46.6±3.5%, p<0.001 to a total of 10.4±0.7% (Figure 3-21a,b,e). SOX2 and Nestin were unchanged. This suggests that treatment with DHA during proliferation only prevents the effect of Cortisol by increasing the percentage of dividing and early and mature neuronal cells above the EtOH control (Ki67 27.6±1.4%, Figure 3-18a; Dcx 16.3±0.3%, Figure 3-19a; MAP2 19.4±0.5%, Figure 3-20a) and almost to the DHA only treatment (Ki67 35.2±0.9%, Figure 3-18a; Dcx 18.5±0.1%%, Figure 3-19a; MAP2 15.2±1.6%, Figure 3-20a), while decreasing apoptosis lower than the EtOH (13.7±0.4%, Figure 3-21a) and DHA only treatment (9.9±0.5%, Figure 3-21a). This indicates that the increase in dividing and neuronal cells is partly based on increased survival.

In the **O-- -CC** group cultures were only pretreated with EPA before Cortisol treatment was started. The percentage of Ki67 cells was significantly increased to a total of 30.6±0.7, 23.2±1.7%, p<0.001 higher compared to the Cortisol group (Figure 3-18a,b,f), so was the percentage of MAP2 positive cells (55.4±16.7%, p<0.01, to total of 12.3%, (Figure 3-20a,b,f). Dcx was not increased (Figure 3-19a,b,f). However the increase in MAP2 positive cells compared to the Cortisol group was still lower than the DHA only (15.2±1.6%, Figure 3-20a) and the EtOH group (19.4±0.5%, Figure 3-20a), indicating that to prevent the effect of Cortisol on neuronal differentiation with DHA, DHA is required during differentiation and not only during proliferation. The percentage of activated Caspase-3 positive cells was significantly decreased by -

33.0±2.1%, p<0.001 to a total of 13.1±0.4% (Figure 3-21a,b,f) which is the level of the EtOH group (13.7±0.4%, Figure 3-21a) but higher than the DHA only treatment (9.9±0.5%, Figure 3-21a). Also the percentage of dividing cells was increased above the EtOH group (27.6±1.4%, Figure 3-18a) but was still below the DHA only group (35.2±0.9%, Figure 3-18a). SOX2 and Nestin were unchanged.

All three treatment conditions, using DHA to prevent the effects of Cortisol on proliferation, differentiation and apoptosis, prevent the effect on dividing cells by increasing their proportion above the vehicle control. During neuronal development and apoptosis the treatment groups gave different results. The **OOO –CC** group prevents the effects of Cortisol by increasing the proportion of dividing cells, early and mature neuronal cells above the level of both DHA only and EtOH control. As under this condition DHA failed to prevent the apoptotic effects of Cortisol, this effect cannot be due to increased survival but is due to an increase in proliferation and differentiation. After treatment during differentiation the effects of DHA on apoptosis during decreasing proliferation (Section 3.4.2.1) disappears, while DHA still impacts on the proportion of dividing and neuronal cells. The **OO- -CC** group prevents the effects of Cortisol regarding dividing, neuronal and apoptotic cells even above the EtOH group. The pre-treatment only group (**O-- -CC**) decreases apoptosis below the EtOH group but fails to increase neuronal differentiation above the EtOH, suggesting that DHA is required during stress (Cortisol treatment) to counter act its effects.

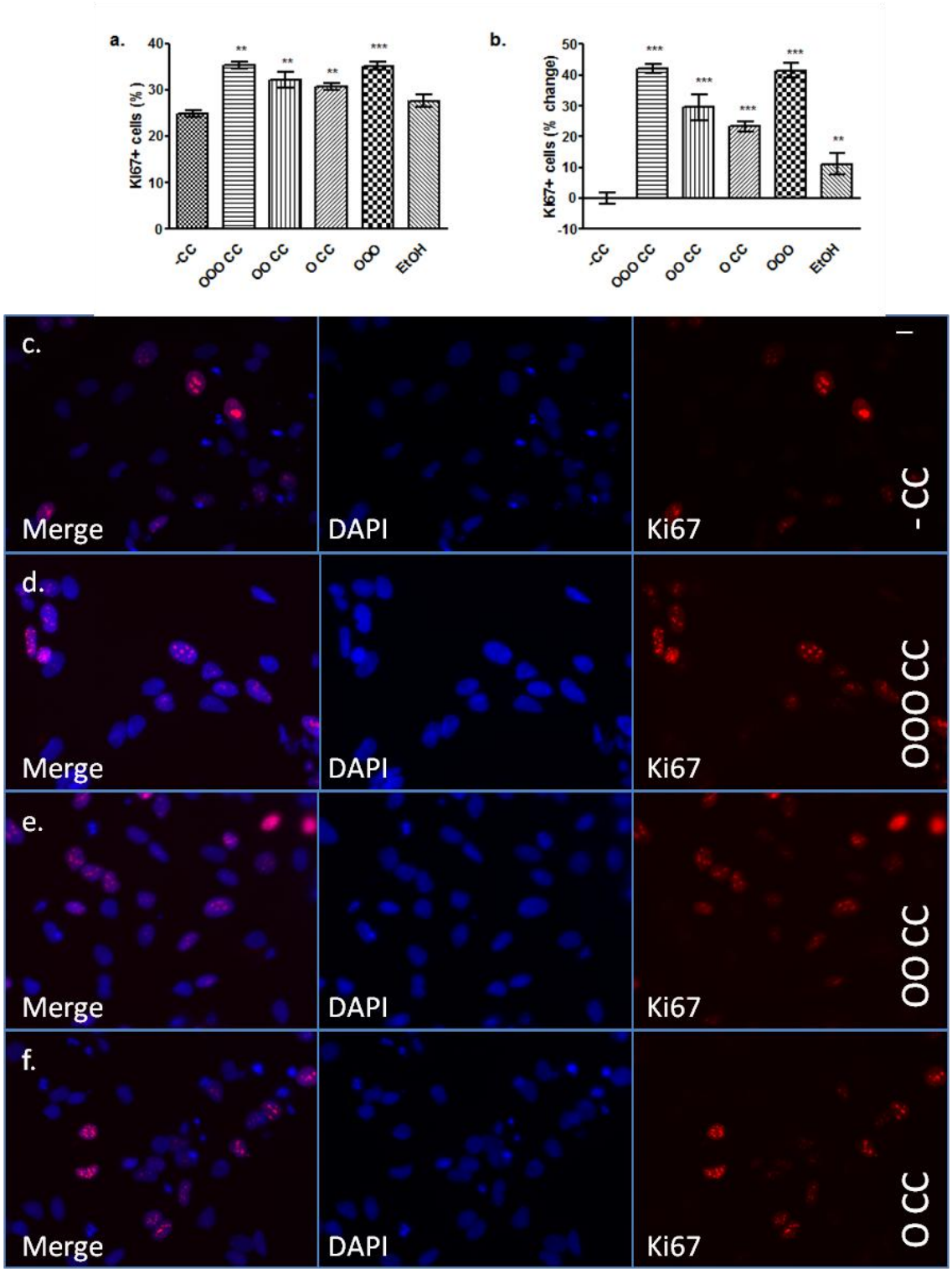


Figure 3-18 Ki67 expression in differentiating HPC03A/07 cells treated with DHA in the Cortisol stress model

a) Comparison of the absolute percentage of Ki67 positive cells. b) Percentage change of Ki67 expression c) Cortisol only; -CC d) continuous DHA treatment before and during Cortisol treatment; OOO –CC e) DHA pre-treatment and treated together with Cortisol during proliferation but not during differentiation, OO- -CC f) DHA pretreatment followed by Cortisol treament. (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

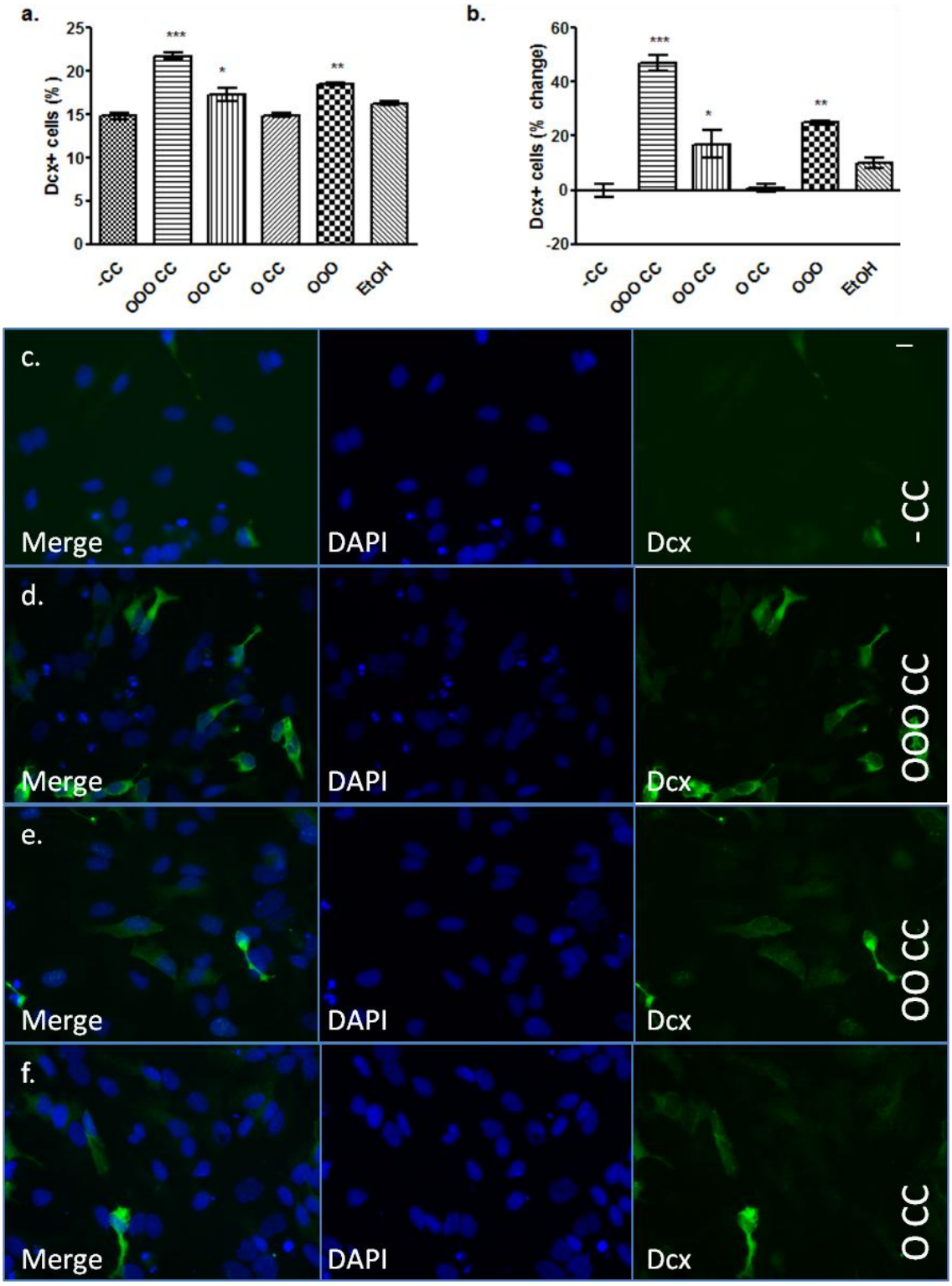


Figure 3-19 Dcx expression in differentiating HPC03A/07 cells treated with DHA in the Cortisol stress model

a) Comparison of the absolute percentage of Dcx positive cells. b) Percentage change of Dcx expression c) Cortisol only; -CC d) continuous DHA treatment before and during Cortisol treatment; OOO –CC e) DHA pre-treatment and treated together with Cortisol during proliferation but not during differentiation, OO- -CC f) DHA pretreatment followed by Cortisol treament. (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

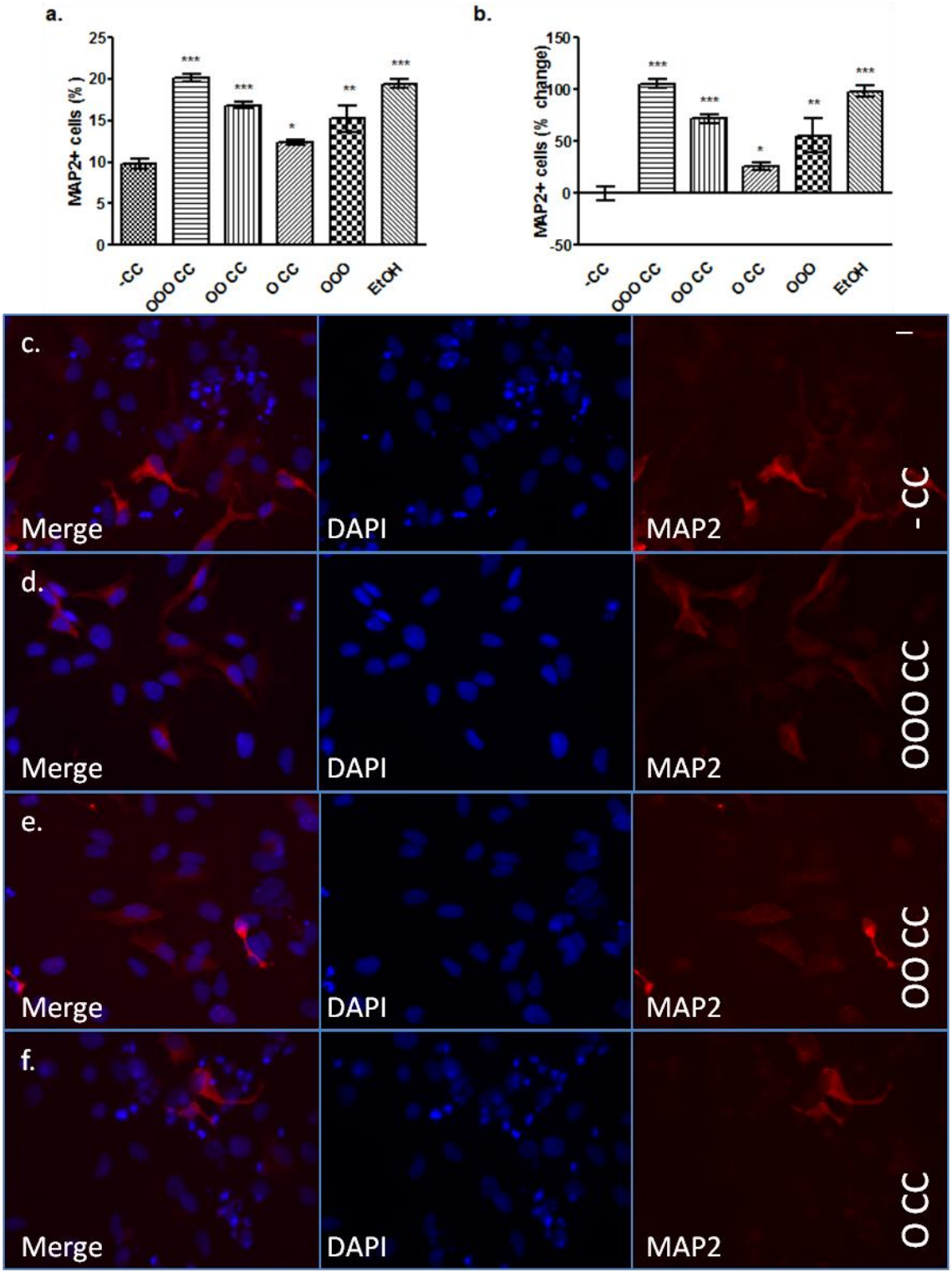


Figure 3-20 MAP2 expression in differentiating HPC03A/07 cells treated with DHA in the Cortisol stress model

a) Comparison of the absolute percentage of MAP2 positive cells. b) Percentage change of MAP2 expression c) Cortisol only; -CC d) continuous DHA treatment before and during Cortisol treatment; OOO –CC e) DHA pre-treatment and treated together with Cortisol during proliferation but not during differentiation, OO- -CC f) DHA pretreatment followed by Cortisol treament. (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

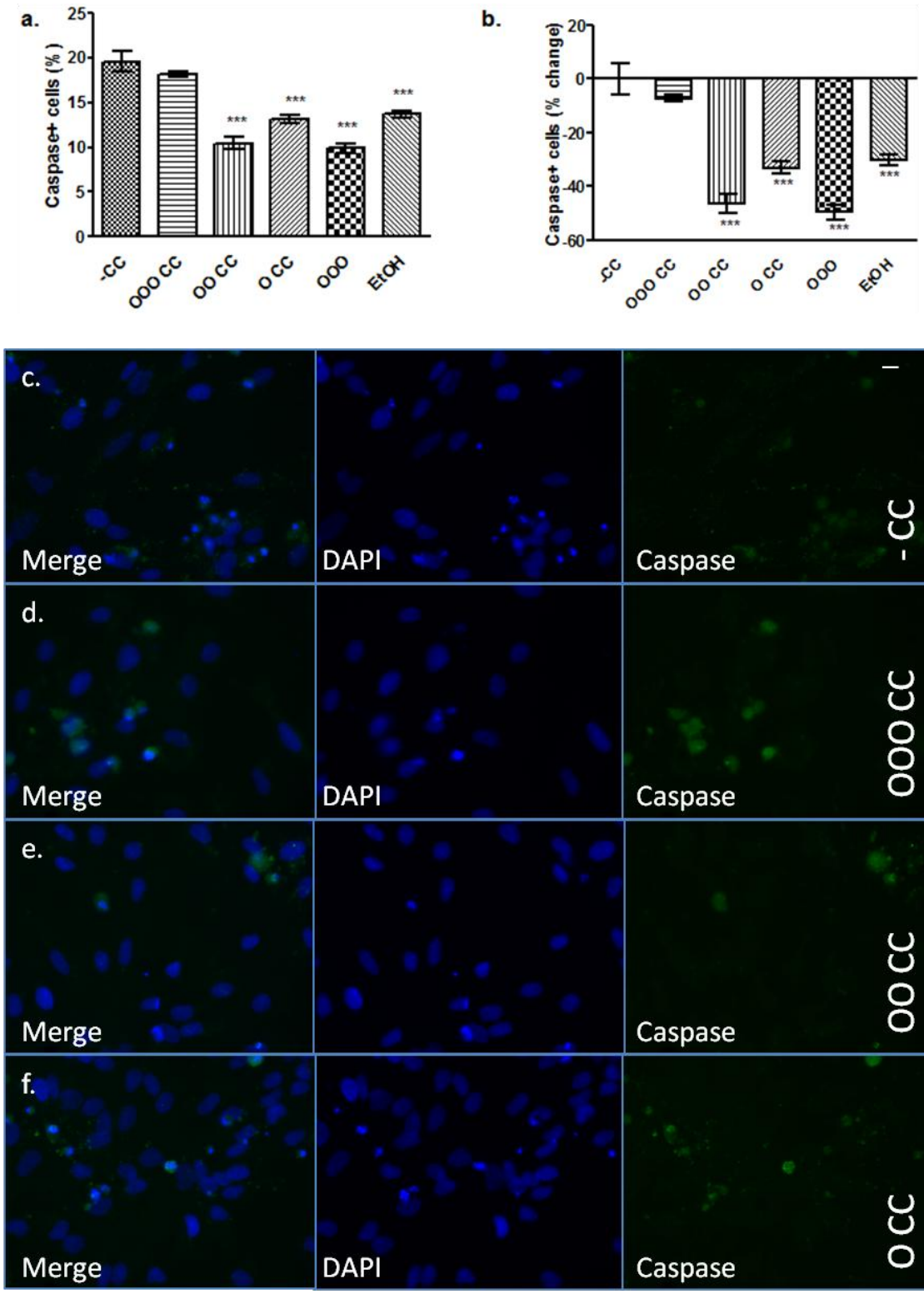


Figure 3-21 Activated Caspase-3 expression in differentiating HPC03A/07 cells treated with DHA in the Cortisol stress model

a) Comparison of the absolute percentage of activated Caspase-3 positive cells. b) Percentage change of activated Caspase-3 expression c) Cortisol only; -CC d) continuous DHA treatment before and during Cortisol treatment; OOO –CC e) DHA pre-treatment and treated together with Cortisol during proliferation but not during differentiation, OO- -CC f) DHA pretreatment followed by Cortisol treament. (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

3.4.3 Preventative effects of Resveratrol on proliferating and differentiating HPC03A/07 cells in a Cortisol Stress Model

In order to assess the effect of RSVL pre-treatment on Cortisol-stressed HPC03A/07 cells, cultures were treated with 1µM RSVL and 100µM Cortisol as described in Section 3.4 and immunocytochemically analyzed to investigate changes in proliferation, differentiation and apoptosis. As shown in Section 0, Cortisol decreases the proportion of dividing and differentiating cells while increasing apoptotic cells over control treatment. I have also shown that RSVL alone increases the proportion of dividing cells and neuroblasts. RSVL has no effect on apoptosis during proliferation and increases it during differentiation (Please see Section 3.2). In the following I am investigating whether RSVL has the potential to prevent the detrimental effects of Cortisol on cell proliferation and neuronal differentiation.

3.4.3.1 RSVL pre-treatment prevents the Cortisol-induced decrease of dividing cells in proliferating HPC03A/07

To investigate the preventative effects of RSVL in the Cortisol stress model in proliferating HPC03A/07, cultures were treated with 1µM RSVL and Cortisol as explained in Section 3.4 / Table 3-2,1. In the **RR –C** group cells were pretreated with RSVL for 3 days and then treated with RSVL and Cortisol together for further 3 days of proliferation. The percentage of Ki67 cells was significantly increased to a total of 38.4±1.1%, 23.9±3.4%, $p<0.01$ higher compared to the Cortisol group, Figure 3-22a,b. The percentage of activated Caspase positive cells was unchanged Figure 3-22c,d. SOX2 or Nestin did not change, please see Section 3.3.

In the **R- -C** group also the percentage of dividing cells was significantly increased compared to the Cortisol group by 16.2±1.4%, $p<0.01$ to a total of 36.0±0.4%, Figure 3-22a,b. The percentage of apoptotic cells (Figure 3-22c,d), SOX2 or Nestin was unchanged.

Both the continuous RSVL treatment and pre-treatment increase the percentage of dividing cells above the EtOH group (34.5±0.9%, Figure 3-22c) but below the RSVL only group (41.9±1.4%). The percentage of activated Caspase positive cells was unchanged and with 17.2±0.8% in the RR –C and in the R- -C group 18.8±0.8% higher than ETOH (10.2±0.2%) and RSVL only (13.9±1.2%) and as high as Cortisol (16.8±0.2%). This suggests that RSVL increases the proportion of dividing cells thereby preventing the decreasing effects of Cortisol, but as expected it has no effect on preventing apoptosis.

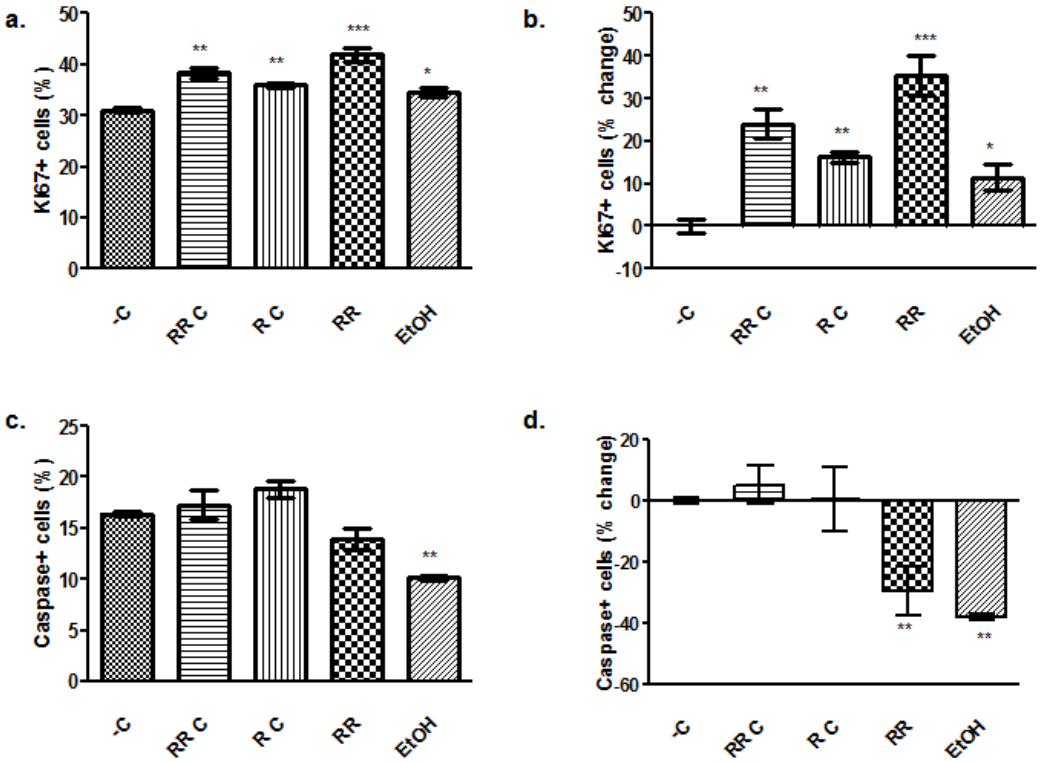


Figure 3-22 Ki67 and activated Caspase-3 in HPC03A/07 cells treated with RSVL under the Cortisol Stress Model

a, c, e, g depict the percentage of positive cells relative to absolute cell numbers, b, d, f, h show the percentage change of the marker in treated cultures compared to Cortisol treated cells. a) and b) Ki67 expressing cells c) and d) activated Caspase-3 expressing cells; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), ***p<0.001; See Table 3-2 for abbreviations

3.4.3.2 RSVL pre-treatment prevents the Cortisol-induced decrease of dividing and neuronal cells in differentiating HPC03A/07

To investigate the preventative effects of RSVL in the Cortisol stress model in differentiating HPC03A/07, cultures were treated with RSVL and Cortisol as described in Section 3.4 / Table 3-2,2). In the **RRR –CC** group cultures were continuously supplemented with RSVL before and during Cortisol treatment the percentage of dividing cells was significantly increased to a total of 34.9±0.2%, 41.3±1.0%, $p<0.001$ increase compared to the Cortisol control (Figure 3-23a,b,d). Also the percentage of Dcx expressing cells (73.2±0.6%, $p>0.001$, total of 27.4±0.1%, Figure 3-24a,b,d) and MAP2 positive cells (MAP2: 158.9±15.7%, $p<0.001$, total of 33.3±0.8%, Figure 3-25a,b,d) was increased. Activated Caspase-3 (Figure 3-26a,b,d), SOX2 and Nestin were unchanged, please see Section 3.3. This shows that under these conditions RSVL increases dividing and early and mature neuronal cells above the EtOH group (Ki67 27.6±1.4%, Figure 3-23a; Dcx 24.4±2.9%, Figure 3-24a; MAP2 24.1±1.0%, Figure 3-25a) and above the RRR group (Ki67 35.2±0.7, Figure 3-23a; Dcx 28.3±1.9%, Figure 3-24a; MAP2 36.7±0.1%, Figure 3-25). This effect appears solely due to an increase in proliferation and differentiation as RSVL treatment has no decreasing effect on apoptosis, indeed RSVL alone increases apoptosis at this concentration (Section 3.2.3) and the co treatment with RSVL does not increase apoptosis above the Cortisol group, Nevertheless the groups with less co treatment display less apoptosis, leading to a treatment response curve, which suggests that the positive effects of RSVL without apoptotic side effects fall in a narrow range. When titrating RSVL in Section 3.2.1 I only examined the effect it has on proliferation and not on apoptosis, however its effects on apoptosis need closer investigation.

RR- -CC cultures were pretreated with RSVL and treated together with Cortisol during proliferation but not during differentiation. The percentage of Ki67 cells in this group was significantly increased to a total of 31.3±0.8%, which is a 27.7±3.4%, $p<0.001$ increase compared to the Cortisol treatment, (Figure 3-23a,b,e). Also, the percentage of Dcx (100.0±3.1%, $p<0.001$, total of 31.7±0.5%, Figure 3-24a,b,e) and MAP2 (MAP2: 68.6±4.7%, $p<0.001$, total of 50.3±3.0, Figure 3-25a,b,e) positive cells was significantly increased. The percentage of Ki67 expressing cells was increased above the EtOH group (Ki67 27.6±1.4%) but below the RRR group Ki67 (35.2±0.7%, Figure 3-23a) and the percentage of neuroblasts and mature neuronal cells was a lot higher than the EtOH (Dcx 24.4±2.9%; MAP2 24.1±1.0%) and RRR group (Dcx 28.3±1.9%; MAP2 36.7±0.1%), See Figure 3-24a Figure 3-25a respectively. Again the percentage of apoptotic cells was not significantly changed and with 26.7±1.1% higher than the EtOH (17.7±1.3%) and the RSVL only group (23.5±1.8%), Figure 3-26a. SOX2 and Nestin are unchanged. This suggests that RSVL treatment during proliferation is sufficient to prevent a decrease in the proportion of neuronal cells. Interestingly, proliferation only treatment is actually more efficient than treating with RSVL throughout proliferation and differentiation. Moreover, the increase in the proportion of dividing cells and neuronal cells is not due to increased survival as RSVL has no decreasing effect on apoptosis but is due to increased proliferation and survival.

In the **R-- -CC** group, cultures were only pre-treated with RSVL, the percentage of dividing cells was significantly increased to a total of 32.6±0.6%, increased by 27.1±2.4%, $p<0.001$ compared to the Cortisol only treatment (Figure 3-23a,b,f). Further, the percentage of neuroblasts (30.4±2.2%, $p<0.05$, total of 20.6±0.4%, Figure 3-24a,b,f) and mature neurons (89.2±0.5%, $p<0.001$, 32.7±0.9%, Figure

3-25a,b,f) was also significantly increased. Activated Caspase-3 (Figure 3-26a,b,f), SOX2 or Nestin were unchanged. This data suggests that pre-treatment only is sufficient to increase the percentage of dividing cells and mature neuronal cells above the EtOH control group (Ki67 27.6±1.4%, Figure 3-23a; MAP2 24.1±1.0%, Figure 3-25a), and the percentage of neuroblasts was higher than the Cortisol group (15.8±0.9%) but lower than the EtOH group (Dcx 24.4±2.9%) (Figure 3-24a). As previously stated a co treatment with Cortisol and RSVL has no effect on apoptosis and RSVL treatment alone already increases apoptosis in differentiating cultures.

Together these results indicate that RSVL treatment in all three groups increases the percentage of dividing and neuronal cells. None of the groups decreases apoptosis making the increase in dividing cells a genuine result that is not due to increased survival. However, RSVL apparently already increases apoptosis on its own and it is necessary to examine which cell types die. The treatment group with RSVL treatment during proliferation together with Cortisol (RR- -CC) shows the highest increase in neuronal proliferation, and pre-treatment only increases mature neuronal cells and neuroblasts compared to the Cortisol group but below the EtOH group. This suggests that pre-treatment alone is sufficient to improve neuronal differentiation compared to the Cortisol treatment but not back to level of EtOH treated cultures. However to increase the percentage of neuronal cells above the level of the EtOH group, RSVL treatment appears to be necessary together with Cortisol during proliferation (RR- -CC) but not during differentiation (RRR –CC).

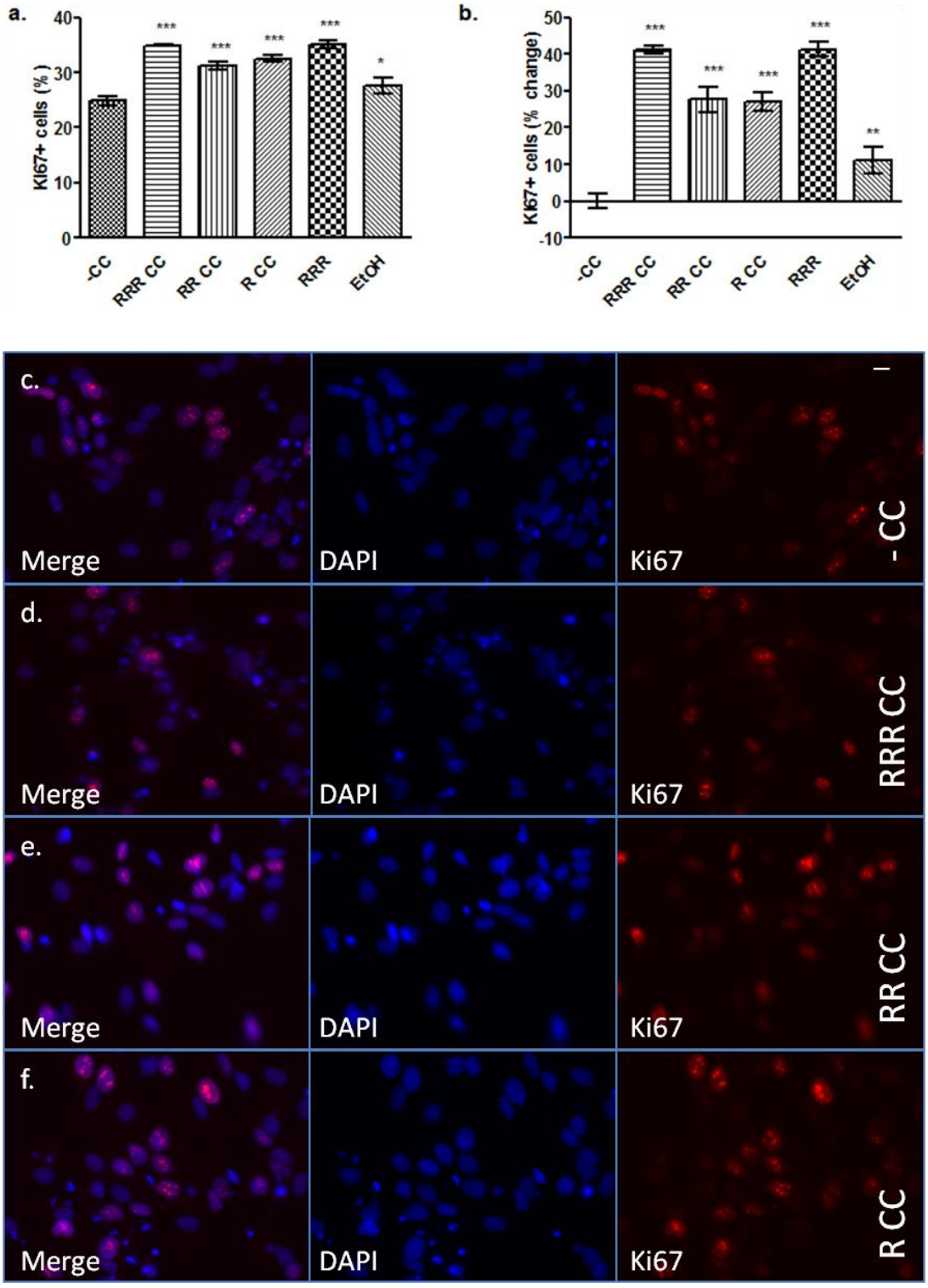


Figure 3-23 Ki67 expression in differentiating HPC03A/07 cells treated with RSVL in the Cortisol stress model

a) Comparison of the absolute percentage of Ki67 positive cells. b) Percentage change of Ki67 expression c) Cortisol only; -CC d) continuous RSVL treatment before and during Cortisol treatment; RRR –CC e) RSVL pre-treatment and treated together with Cortisol during proliferation but not during differentiation, RR- -CC f) RSVL pretreatment followed by Cortisol treament. (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

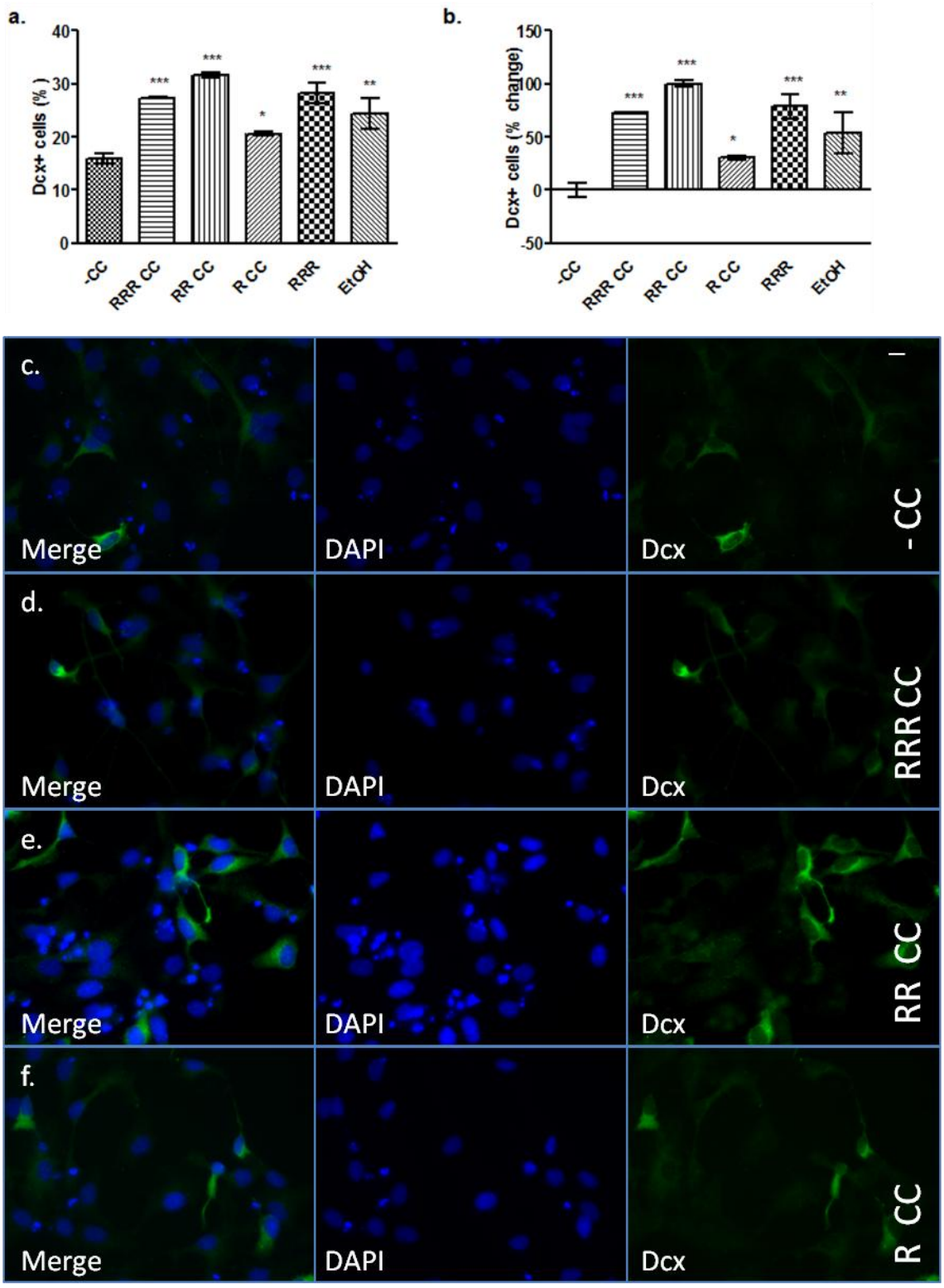


Figure 3-24 Dcx expression in differentiating HPC03A/07 cells treated with RSVL in the Cortisol stress model

a) Comparison of the absolute percentage of Dcx positive cells. b) Percentage change of Dcx expression c) Cortisol only; -CC d) continuous RSVL treatment before and during Cortisol treatment; RRR –CC e) RSVL pre-treatment and treated together with Cortisol during proliferation but not during differentiation, RR- -CC f) RSVL pretreatment followed by Cortisol treament. (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

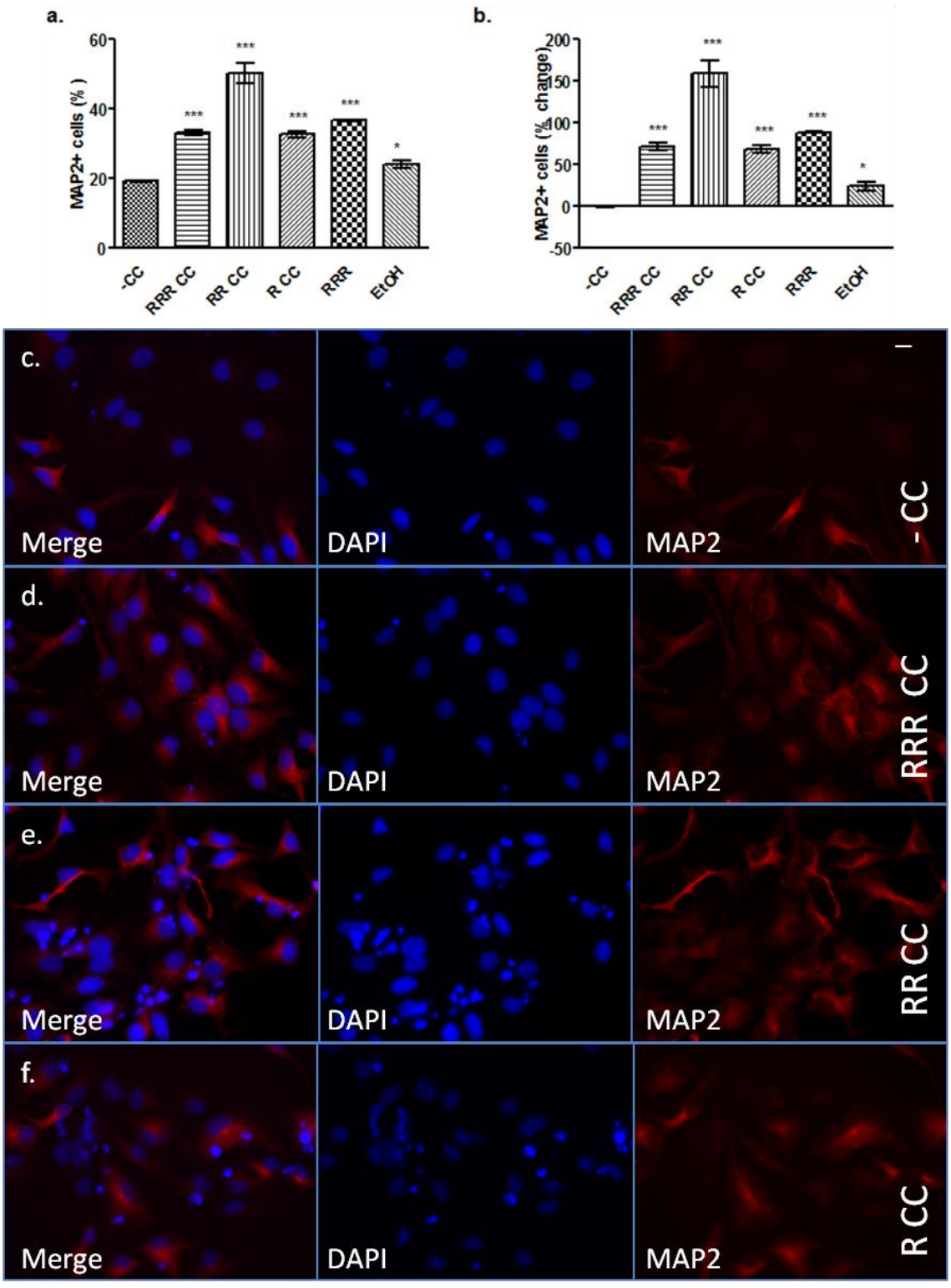


Figure 3-25 MAP2 expression in differentiating HPC03A/07 cells treated with RSVL in the Cortisol stress model

a) Comparison of the absolute percentage of MAP2 positive cells. b) Percentage change of MAP2 expression c) Cortisol only; -CC d) continuous RSVL treatment before and during Cortisol treatment; RRR –CC e) RSVL pre-treatment and treated together with Cortisol during proliferation but not during differentiation, RR- -CC f) RSVL pretreatment followed by Cortisol treament. (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

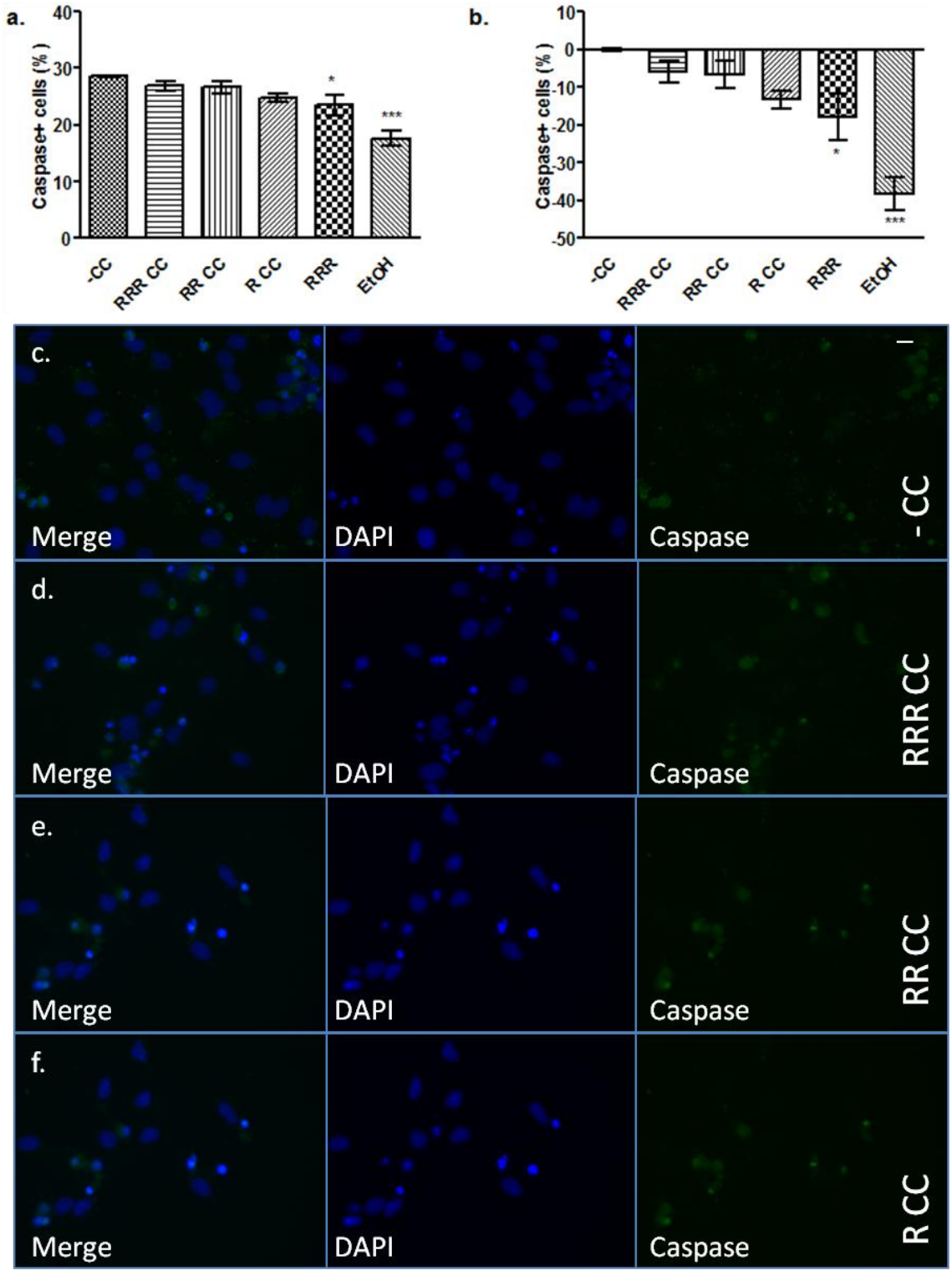


Figure 3-26 Activated Caspase-3 expression in differentiating HPC03A/07 cells treated with RSVL in the Cortisol stress model

a) Comparison of the absolute percentage of activated Caspase-3 positive cells. b) Percentage change of activated Caspase-3 expression c) Cortisol only; -CC d) continuous RSVL treatment before and during Cortisol treatment; RRR –CC e) RSVL pre-treatment and treated together with Cortisol during proliferation but not during differentiation, RR- -CC f) RSVL only (Scalebar: 20µm; p-values were generated using One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001; See Table 3-2 for abbreviations)

3.4.4 Summary

Table 3-3 summarizes the results from the EPA, DHA and RSVL prevention experiments in the Cortisol stress model in proliferating HPC03A/07 cells, Table 3-4 summarizes the results in differentiating HPC03A/07 cells.

The Omega-3s EPA and DHA and the stilbenoid RSVL display differences in their ability to prevent the effects of Cortisol, namely decreasing dividing and neuronal cells while increasing apoptosis. All components can prevent those Cortisol induced effects although with differences in the subgroups. RSVL is different from EPA and DHA as it does not decrease apoptosis and even slightly increases it when used on its own. EPA and DHA during proliferation increases the proportion of dividing cells despite Cortisol treatment in both groups, pre-treatment only and continuous treatment, above the level of the vehicle control and EPA even to the level of EPA only treatment, whereas DHA did not reach the level of the DHA only group. Both EPA and DHA were able to decrease the proportion of apoptotic cells below the vehicle control and this time DHA reached the level of the DHA only treatment but not EPA (Table 3-3). During differentiation (Table 3-4), EPA treatment in all three groups, continuous EPA treatment (OOO –CC), EPA treatment during proliferation together with Cortisol (OO- -CC) and pre-treatment only (O-- -CC) has a very similar effect on increasing the proportion of dividing and neuronal cells and in decreasing apoptotic cells. The exception is the proportion of neuroblasts in the continuous treatment group that was not increased. This suggests that EPA treatment during differentiation pushes neuronal differentiation and maturation and after 7 days all Dcx positive cells have matured. DHA performs very differently in the three subgroups, it appears that pre-treatment only is sufficient for preventing a decline in dividing cells; however it is not sufficient for neuronal differentiation

and maturation. Not only is the increase greater when cells a treated with DHA during proliferation in the Cortisol stress model but it even appears to be required during differentiation to ensure an increase in neuroblasts and mature neurons above or to the level of the vehicle control respectively. Both EPA and DHA decrease apoptosis in all three subgroups similarly compared to the Cortisol group. It appears that in the Cortisol stress model EPA is rather required for cell division and survival as EPA pre-treatment is already sufficient to achieve neuronal differentiation and maturation, whereas DHA is required to be present during the stress mimicking Cortisol treatment during proliferation and differentiation to ensure neuronal differentiation.

RSVL treatment during proliferation increases the proportion of dividing cells most efficiently when present continuously, however not the level of the RSVL only treatment (Table 3-3). RSVL has no preventing effect on stress-induced apoptosis, neither during proliferation nor during differentiation. During differentiation (Table 3-4) pre-treatment only appears to be enough to increase dividing and neuronal cells, both early and mature, to almost the vehicle level, however when cultures were treated also during proliferation once the Cortisol treatment was started the increase was even greater although not to the level of RSVL only but above the vehicle. Continuous RSVL treatment also during differentiation increased dividing and neuronal cells; however not as efficiently as when cultures were treated during proliferation only.

3 d pro				
EPA	**OO C**	**O C**	**OO**	**EtOH**
Ki67	↑↑↑	↑↑↑	↑↑↑	↑↑
Caspase	↓	↓	↓↓	↓
SOX2	-	-	-	-
Nestin	-	-	-	-
DHA	**OO C**	**O C**	**OO**	**EtOH**
Ki67	↑	↑	↑↑↑	↑↑
Caspase	↓↓	↓↓	↓↓	↓↓
SOX2	-	-	-	-
Nestin	-	-	-	-
RSVL	**RR C**	**R C**	**RR**	**EtOH**
Ki67	↑↑	↑↑	↑↑↑	↑
Caspase	-	-	-	↓
SOX2	-	-	-	-
Nestin	↓	-	-	-

Table 3-3 Summary of the effect of EPA, DHA, RSVL on HPC03A/07 during proliferation under the Cortisol stress model.
↑ stands for significanlty increased expression; ↓ for significantly decreased expression; - for no significant changes. Changes are relative to Cortisol treated cultures. DHA: Docosahexaenoic acid, EPA: eicosapentaenoic acid; RSVL: Resveratrol; Pro: Proliferation; Diff: Differentiation

7d diff					
EPA	**OOO CC**	**OO CC**	**O CC**	**OOO**	**EtOH**
Ki67	↑	↑↑	↑	↑↑	↑
Dcx	-	↑	↑	↑	-
MAP2	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
Caspase	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
SOX2	-	-	-	-	-
Nestin	-	-	-	-	-
DHA	**OOO CC**	**OO CC**	**O CC**	**OOO**	**EtOH**
Ki67	↑↑	↑↑	↑↑	↑↑↑	↑
Dcx	↑↑↑	↑	-	↑↑	-
MAP2	↑↑↑	↑↑↑	↑	↑↑	↑↑↑
Caspase	-	↓↓↓	↓↓↓	↓↓↓	↓↓↓
SOX2	-	-	-	-	-
Nestin	-	-	-	-	-
RSVL	**RRR CC**	**RR CC**	**R CC**	**RRR**	**EtOH**
Ki67	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑
Dcx	↑↑↑	↑↑↑	↑	↑↑↑	↑↑
MAP2	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑
Caspase	-	-	-	↓	↓↓↓
SOX2	-	-	-	-	-
Nestin	-	-	-	-	-

Table 3-4 Summary of the effect of EPA, DHA, RSVL on HPC03A/07 during differentiation under the Cortisol stress model.
↑ stands for significanlty increased expression; ↓ for significantly decreased expression; - for no significant changes. Changes are realative to Cortisol treated cultures. DHA: Docosahexaenoic acid, EPA: eicosapentaenoic acid; RSVL: Resveratrol; Pro: Proliferation; Diff: Differentiation

3.5 Discussion

3.5.1 Omega-3 fatty EPA and DHA acids increase the percentage of dividing cells and neurogenesis while decreasing apoptosis

My results show that the Omega-3 fatty acids EPA and DHA increase the proportion of dividing cells and neuronal cells and decreases apoptosis *in vitro* in HPC03A/07 cells (3.1), suggesting that EPA and DHA promote neurogenesis and the percentage of dividing cells mainly by increased survival but also due to increased neurogenesis. DHA effectively promotes neurogenesis and suppresses apoptosis both *in vitro* and *in vivo* in rat, suggesting that it modulates hippocampal function via neurogenesis (Kawakita et al., 2006). In adult immune stressed mice a diet enriched with PUFA decreased hippocampal microglia activation and increased proliferation (BrdU) and neurogenesis (Dcx) in the hippocampus leading to normal long-term potentiation (Crupi et al., 2011). This increase in proliferation and neurogenesis as well as the alleviation in decreased hippocampal Dcx expression by EPA and DHA in old rats (Dyall et al., 2010) are in accordance with my data, suggesting a positive effect of EPA and DHA on neurogenesis partly due to an increase in survival but also due to increased neurogenesis. The decrease in apoptotic cells is smaller than the combined increase in dividing cells (Ki67), neuroblasts (Dcx) and mature neurons (MAP2).

In the literature to date, it is well accepted that the Omega-3 fatty acids EPA and DHA are crucial for the development of a healthy brain and are necessary throughout adulthood to maintain the normal brain function required for learning and memory, such as neurogenesis, synaptic plasticity and neuronal protection (Makrides et al., 2010). Interestingly, populations with high fish consumption

show a lower prevalence of major depression (Hibbeln, 1998; van Gelder et al., 2007). And a deficiency of hippocampal Omega-3s is associated with a decrease in learning and memory abilities in rodents and humans (Freemantle et al., 2006; Chung et al., 2008) and can be reversed with a supplemented diet. Omega-3 supplemented diet enhances working and reference memory in healthy control animals and improves in Omega-3 fatty acid deprived rats. Further DHA is accumulated especially in the hippocampus and the olfactory bulb compared to visual cortex and cerebellum (Chung et al., 2008), suggesting that a Omega-3 supplemented diet leads to an accumulation of DHA areas with ongoing neuronal differentiation and maturation in the rodent brain and enhances learning and memory also in healthy animals. This raised the question whether pre-treatment with Omega-3 fatty acids may be able to prevent the effects of stress-relevant high concentrations of Cortisol.

My results in 0 showed that high concentrations of the endogenous glucocorticoid hormone Cortisol (100µM) decrease the proportion of dividing cells and neurogenesis but increase apoptosis in HPC03A/07. The decrease in proliferation and neurogenesis in my results are partly due to an increased apoptosis and partly due to a genuine decrease in cell division and neurogenesis. This is because the increase in the percentage of cell death is smaller than the combined decrease in the percentage of dividing cells and neuronal cells. Anacker et al., using the same cell line and Cortisol concentration, showed that Cortisol only decreases proliferation and neurogenesis when cells are treated during the mitotic phase (Anacker et al., 2011), which is why I treated the cultures with Cortisol under proliferation conditions before starting differentiation. Anacker et al. showed as well that Cortisol treatment does not affect apoptosis, whereas my data showed an

increase in apoptosis. However he treated the cells for only 24h under proliferative conditions before starting 7 days of differentiation, whereas I treated them for 3 days of proliferation and 7 days of differentiation, treating them during differentiation as well. High levels of glucocorticoids have been shown to induce cell death in lymphoid cells and rat brain (Gould et al., 1991; Haynes et al., 2003; Miller et al., 2005). The increase in apoptosis in my experimental setting can be explained by the longer exposure to Cortisol.

3.5.2 Pre-treatment with EPA and DHA can prevent the negative effect of stress relevant concentrations of Cortisol (100µM) on dividing cells, neurogenesis and apoptosis

My results in 3.4 showed that pre-treatment with either EPA or DHA could prevent the detrimental effects high Cortisol concentrations have on HPC03A/07: decrease of proliferation and neurogenesis that are partly due to decreased survival but also partly a genuine decrease in proliferation and neurogenesis. This can be explained by the greater combined increase of the dividing and differentiating cells compared to the apoptotic cells.

These results are in line with the results of a study in adult immune stressed mice. In these mice a diet enriched with PUFA decreased hippocampal microglia activation and increased neurogenesis in the hippocampus leading to normal long-term potentiation (Crupi et al., 2011). This demonstrates that Omega-3s prevent neuroinflammation and deficits of hippocampal plasticity in immune stressed mice. Increased immune stress occurs in neuropsychiatric diseases associated with autoimmune diseases and the preventative properties described above suggest that Omega-3 treatment could act as a potential therapeutic option. Moreover DHA reduces cytokine-induced expression of

proatherogenic and proinflammatory proteins in human endothelial cells. De Caterina et al. conducted a similar pre-treatment experiment in human endothelial cells: Cells were pretreated with 10µM DHA and EPA followed by stimulation with TNFα and Il-1. Endothelial cells pretreated with DHA but not EPA had decreased expression of the endothelial leukocyte adhesion molecules vascular cell adhesion molecule (VCAM-1), intracellular adhesion molecule (ICAM-1) and e-selectin (De Caterina et al., 1994). These molecules are required for the adhesion of leukocytes to the endothelium and their migration in endothelial inflammation and atheroma formation. A decrease in inflammatory proteins and subsequent atherosclerosis prevention can prevent inflammation and secure and improve the blood supply to the brain. A decreased blood flow to the brain is associated with dementia, known to deteriorate with age and is vital for optimal brain function (Nagahama et al., 2003; Ruitenberg et al., 2005). De Caterina's, Crupis and my results show that EPA and DHA have preventative properties in endothelial cells, in mouse brain and in human hippocampal progenitor cells; however it becomes apparent that EPA and DHA have different functions in the brain and the cardiovascular system. I will further discuss the differences between EPA and DHA below.

My results are further in line with the literature to date that support the neurogenesis hypothesis of depression and cognition, which postulates that increased neurogenesis in the dentate gyrus of the hippocampus in the adult brain, is required to improve depressive symptoms and learning and memory. I could show that Omega-3 FAs have a similar effect on neurogenesis HPC03A/07 cells as antidepressants (Anacker et al., 2011). Literature to date has demonstrated that the by elevated corticosterone levels in the dentate gyrus of adult mice induced behavioural deficits and the decrease in cell

proliferation are reversed by chronic monoaminergic antidepressant (for example, fluoxetine) treatment (Murray et al., 2008). And when hippocampal neurogenesis is abolished by X-irradiation, the efficacy of fluoxetine is blocked in some, but not all, behavioural paradigms, suggesting both neurogenesis dependent and independent mechanisms of antidepressant action (David et al., 2009). Antidepressant treatments have been shown to increase proliferation of NPC, neurogenesis and survival of neurons in the adult brain, especially the hippocampus (Jacobs et al., 2000; Malberg et al., 2000; Duman, 2004; Wang et al., 2008; Boldrini et al., 2009). These effects are dependent on the mouse strain but are always most pronounced in previously stressed animals (David et al., 2010). Stressors such as high levels of corticosterone might enhance the potency of the treatment such as monoaminergic antidepressants or Omega-3 fatty acids. These treatments might even only elicit their positive effect if a stress or disease induced deficit prevails (David et al., 2010). This might explain the results I observed in the DHA treatment group under differentiation conditions (3.4.2.2): cultures that have been pre-treated, then continuously treated with DHA together with Cortisol treatment (OOO –CC), showed significantly increase in early and mature neurons compared to the vehicle treated and even the DHA only treated group. On the other hand the proportion of apoptotic cells is not decreased compared to Cortisol only treatment and is still increased compared to the vehicle control. This could indicate that continuous DHA treatment pushes HPC03A/07 cells into fate commitment and differentiation without preventing the Cortisol induced effects on apoptosis. EPA treatment in turn increased the proportion of differentiating cells, however never beyond the level of the EPA only treated cultures. However, these results also suggest that DHA, not EPA, is required during proliferation and differentiation

for neuronal differentiation (Dcx) and maturation into MAP2 positive cells in the Cortisol stress model. Anacker et al. demonstrated that the effects of the antidepressant sertraline on proliferating progenitor cells was essential to induce NPC differentiation into Dcx-positive neuroblasts, but not sufficient to promote their maturation into MAP2-positive neurons, because the number of MAP2-positive neurons was only increased when cells were treated continuously during proliferation and differentiation. Treatment during differentiation only had no effect (Anacker et al., 2011). Similar to this experiment the different effects of Omega-3 treatment on NPC differentiation in HPC03A/07 cells treated only during proliferation and treatment during differentiation only needs to be investigatet.

3.5.3 EPA pre-treatment enhances survival and differentiation of HPC03/A07 cells in the Cortisol stress model while DHA is required for neuronal maturation and enhances survival

As stated above my results in this thesis (3.4) show that EPA and DHA prevent the effects of Cortisol, namely decreasing proliferation and differentiation partly due to decreased survival, in my *in vitro* stress model. DHA exerts its preventative effect during both the proliferation and differentiation phase to ensure neuronal differentiation, while EPA pre-treatment is sufficient to prevent the Cortisol-induced effects on dividing cells, neuroblasts and mature neurons.

Interestingly, in this thesis for the first time human hippocampal stem cells were used to investigate the effects of EPA and DHA, not in a mix but separately, on cell division and differentiation. My results show distinct differences in the effect of EPA and DHA. To date, most studies have used a mix of both EPA and DHA and so could not study individual effects of EPA and DHA. Two meta-analyses

started to examine the differences in efficacy and mode of action of EPA and DHA on depression and cardiovascular risk factors. EPA may be more efficacious than DHA in treating depression (Martins, 2009) whereas DHA appears to be more efficient in decreasing blood pressure, heart rate and platelet aggregation compared to EPA (Cottin et al., 2011). However both came to the conclusion that further evidence is required to fully understand those differences. An example for how difficult it is to compare the results from different groups on the differences between EPA and DHA is shown here: Two groups assess the impact of EPA and DHA on leukocyte function *in vivo*, but use different concentrations and use different means to assess their outcome. One group reported that DHA but not EPA supplementation decreased T-lymphocyte activation, which was assessed by CD69 expression (Kew et al., 2004) whereas another group (Gorjao et al., 2006) showed an increased lymphocyte proliferation under DHA treatment. This kind of discrepancy makes it very difficult to compare research from different groups.

My results show that EPA and DHA treatment has some similar effects and also distinctly different effects on HPC03A/07 cells. EPA and DHA treatment under proliferative conditions increases the percentage of dividing cells despite Cortisol treatment in the pre-treatment only (O- -C) and the continuous treatment group (OO –C). Please see 3.4 for a detailed description of the treatment groups. Both increase the proportion of dividing cells above the level of the vehicle control and EPA even to the level of EPA only treatment, whereas DHA did not reach the level of the DHA only group. Both EPA and DHA were able to decrease the percentage of apoptotic cells below the vehicle control, DHA to the level of the DHA only treatment but not EPA. This suggests that under proliferative conditions in HPC03A/07 cells EPA is better in preventing

the effects Cortisol has on dividing cells, suggesting an effect on cell division; whereas DHA can better prevent the Cortisol induced effects on apoptosis in proliferating cells, increasing the survival of NPC (See 3.4.1 and 3.4.2).

During differentiation in the Cortisol stress model, EPA treatment in all three groups: continuous EPA treatment (OOO –CC), EPA treatment during proliferation together with Cortisol (OO- -CC) and pre-treatment only (O-- -CC), (see 3.4.1.2) has a very similar effect on increasing the proportion of dividing cells, neuroblasts (Dcx) and mature neuronal cells (MAP2) and in decreasing apoptotic cells. The exception is the percentage of neuroblasts (Dcx) in the continuous treatment group was not increased; however MAP2 positive mature neurons were increased. This suggests that EPA treatment during differentiation pushes neuronal differentiation and maturation and after 7 days all Dcx positive cells have already matured. DHA, on the other hand, behaves very differently in the three subgroups; it appears that pre-treatment alone is sufficient to prevent a decline in dividing cells mainly due to increased survival. However this is not sufficient to overcome the effects of Cortisol on neuronal differentiation and maturation. Not only is this increase in dividing cells greater when cells are treated with DHA during proliferation in the Cortisol stress model (OO- -CC), but it even appears to be required during differentiation (OOO –CC) to overcome the effect of Cortisol and ensure an increase in neuroblasts and mature neurons above or to the level of the vehicle control, see 3.4.2.2. Both EPA and DHA decrease apoptosis in all three subgroups similarly compared to the Cortisol group and prevent the Cortisol induced increase in apoptosis. Except under DHA treatment in the continuous treatment group as discussed in Section 3.5.2. It appears that in the Cortisol stress model, EPA is increasing cell survival and EPA pre-treatment is already sufficient to achieve neuronal

differentiation and maturation. Conversely, DHA is required to be present throughout proliferation and differentiation to overcome the effects of Cortisol and ensure neuronal differentiation and maturation. This is not only due to increased survival as the proportion of apoptotic cells in not decreased. It might be possible that under EPA treatment at an earlier time point in differentiation (3 days) a significant increase of Dcx positive neuroblasts could occur and that after 7 days these neuroblasts have already matured into MAP2 positive neurons. Whereas cell differentiation into mature neurons under DHA treatment might take longer and the proportion of MAP2 positive cells might only increase after e.g. 14 days under differentiation conditions. On the other hand the survival of the neuroblasts or the mature neurons might not be maintained during DHA treatment, and the cells might die on their way to becoming mature neurons. This holds also true for the EPA or DHA only treated group. Therefore it will be necessary to characterise the nature of the apoptotic cells to explore what cell types die. Assessing the effects of EPA and DHA at different time points, for example, 3 and 14 days of differentiation would be an interesting experiment to conduct to investigate how Dcx, MAP2 and activated Caspase 3 expression change over time.

Due to the differences in acyl chain length and degree of saturation EPA (20:5 n-3) and DHA (22:6 n-3) would not be expected to have the same effect on cell structure and signalling and therefore brain function. However, the heterogeneity of studies investigating the effects of EPA and DHA in terms of dosage, duration, population target, cell line, sample size, as well as the relative amount of EPA and DHA used in supplements leads to variability in results in *in vivo* and *in vitro* studies, making it difficult to pin point differences between the modes of action of these two main Omega-3s. In the following section I am

going to discuss the data to date that compares EPA and DHA regarding brain content, inter-conversion, impact on depression, cardiovascular system, inflammation, gene expression and signalling to find possible explanations for the differences between EPA and DHA in my results.

DHA is predominately found in the phophatidylethanolamine (PE) and the phosphatidylserin (PS) fraction which together with phophatidylcholin (PC) constitute the major building blocks of neuronal membranes (Rapoport, 2001). Stable isotope tracer studies showed that increasing ALA intake for some weeks to months elevates EPA but not DHA in plasma lipids, erythrocytes, leukocytes, platelets and breast milk (Burdge and Calder, 2005). ALA, EPA and DHA can be inter-converted although only to a minimal extent all the way through to DHA (Burdge and Calder, 2006). This explains the importance of sufficient dietary DHA intake. DHA supplementation has been shown to increase neurogenesis *in vivo* in rats and *in vitro* (Kawakita et al., 2006). However, a meta-analysis by Martins showed that EPA or a combination with a higher EPA to DHA ratio is more efficacious in alleviating depressive symptoms in human trials (Martins, 2009). My data illustrate that DHA supplementation in the Cortisol stress model is required to improve neuronal differentiation and maturation, whereas EPA pre-treatment is sufficient to prevent the Cortisol induced effects on proliferation and differentiation mainly by increasing survival (shown in 3.4) Furthermore, EPA seams more important for the survival of dividing cells (3.4.1.1). It might be that EPA is more efficacious in human trials because it is more relevant for proliferation and later can be converted to DHA. Indeed, the enzymes necessary for this conversion have been shown in my lab to be present in the HPC03A/07 cell line on mRNA level. DHA also might get degraded more easily on the way to the brain due to its length and additional

double bond. Regarding perinatal depression, a meta-analysis did not show any significant differences between the effect of EPA and DHA on depression, although most of the trials were of poor quality due to small sample sizes and failure to adhere to Consolidated Standards of Reporting Trials guidelines (Jans et al., 2010). Contrary to these findings a recent meta-analysis by Cottin et al. analysed the differential effects of EPA and DHA on cardiovascular risk factors and showed that DHA and not EPA appears to be responsible for lowering blood pressure and heart rate as well as platelet function (Cottin et al., 2011). An improved cardiovascular system can be beneficial for brain functions as increased blood flow in the brain is known to facilitate AHN (Nagahama et al., 2003; Ruitenberg et al., 2005). Additionally, fMRI studies have shown that cerebral blood flow is decreased in patients with dementia (Nagahama et al., 2003; Ruitenberg et al., 2005). Fish oil consumption is also beneficial for arterial compliance and endothelial function (Cottin et al., 2011). However, any differences between EPA and DHA regarding their vascular effect are poorly understood. EPA and DHA also markedly affected the expression of genes clustered as cytokines and related receptors, signal transduction pathways, transcription factors, cell cycle, defence and repair, apoptosis, DNA synthesis, cell adhesion, cytoskeleton, and hormone receptors in T-Lymphocytes. Noticeable differences were observed between the effects of EPA and DHA, indicating that it is an over-simplification to generalize the effects of Omega-3s. For a detailed review on changes in gene expression please see (Verlengia et al., 2004). Although *in vitro* and *in vivo* studies indicated an effect on inflammation or insulin sensitivity, neither EPA nor DHA alone showed an effect in human subjects (Cottin et al., 2011). EPA and DHA also impact on the mitogen activated protein kinase (MAPK) pathway that modulates cellular

activities, such as proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis. Xue at al. observed differences in EPA and DHA on the MAPK pathway in human umbilical vein cells (HUVEC) that were activated with TNF-α (Xue et al., 2006). EPA or DHA alone significantly reduced the TNF-α-induced activation of p38 and JNK kinases, but EPA is a more potent inhibitor than DHA. Further, DHA and not EPA reduced the TNF-α-induced JNK mRNA expression, suggesting that EPA and DHA can inhibit stress induced apoptosis and inflammation. These data could explain the differences between EPA and DHA in neurogenesis in the continuous treatment group (OOO –CC) of the Cortisol stress model where EPA increases neuroblasts and mature neurons while DHA increases mature neurons only when cells were treated during differentiation (see 3.4) and the differences between EPA and DHA only in neurogenesis and the degree of decrease in apoptosis observed where EPA alone increases mature neurons while DHA increases neuroblasts. EPA alone decreases apoptosis to a greater level than DHA. EPA as the more potent inhibitor of JNK kinases and p38 might facilitate the proper maturation of neuroblasts short-term, whereas DHA is a long-term inhibitor by reducing the JNK kinase mRNA (Xue et al., 2006) and might therefore be more important for differentiation and maturation. Further supporting the idea that DHA is more effective in easing inflammatory stress is provided by Weldon et al. who in an *in vitro* study indicated that DHA may be more effective than EPA in alleviating lipopolysaccharide induced pro-inflammatory cytokine production by macrophages (Weldon et al., 2007). The proinflammatory cytokines tumour necrosis factor α (TNFα) and interleukin (IL)-6 are found to be increased in patients with Major depressive disorder (MDD) (Dowlati et al., 2010; Liu et al., 2011). One explanation for the decrease in cytokines under DHA treatment is

that EPA competes with the Omega-6 FA AA and acts as a substrate for cyclooxigenase and lipoxygenase, being converted to eicosanoids, whereas DHA does not (see 1.3.1). Further, a diet enriched with Omega-3 PUFA dampened the inflammatory response and the physiological re-equilibration of body fat deposition in obese Zucker rats by reducing substrates for inflammatory molecules and endocannabinoids in the visceral adipose tissue and in the liver and heart (Batetta et al., 2009).

EPA and DHA have different effects on membrane structure (Mitchell and Litman, 1998). The length and the degree of unsaturation of fatty acid chains impacts on membrane fluidity as unsaturated lipids due to their double bonds create a kink, preventing the fatty acids from packing together as tightly. Fatty acids decrease membrane organisation with increasing unsaturation from one to six carbon-carbon double bonds (Mitchell and Litman, 1998), which results in increased aqueous defects and therefore increased proton permeability through proton passive pathways (Decoursey, 2003) and a decrease in melting temperature and therefore fluidity. DHA possesses a longer carbon chain and one more double bond than EPA, which is thought to be the reason for the greater influence of DHA on membrane fluidity and cholesterol content (Hashimoto et al., 1999), and thus on the activity of membrane protein or ion channels. Lipid raft content was altered in lymphocytes after DHA but not EPA treatment (Ma et al., 2004). Further, the fatty acid distribution in biological membranes can modulate those membranes and incorporate microdomains, such as lipid rafts and caveolae (Ma et al., 2004). This re-organisation of membrane structures influences cell signalling by moving signalling protein in and/or out of rafts (Wassall and Stillwell, 2008). The IL-2 receptor is located in lipid rafts (Li et al., 2005) and DHA and EPA decrease the stimulatory effect IL-2

has on human lymphocyte proliferation via different pathways: The effect of DHA on lymphocyte proliferation was associated with a reduction in the IL-2-induced activation of the JAK/STAT, ERK, and Akt pathways and a decrease in CD25 expression whereas EPA decreased the phosphorylation of the IL-2R signalling proteins by a mechanism that did not involve CD25 (Gorjao et al., 2007). In MDD patients, levels of IL-2 receptor, TNF-α and IL-6 were significantly higher than controls (Liu et al., 2011), suggesting EPA and DHA as a treatment as they can diminish the increase of inflammatory cytokines as described above.

Two nuclear receptors important for neuroprotection are nuclear retinoid X receptor (RXR) and peroxisome proliferator-activated receptors (PPARs). EPA and DHA are endogenous ligands of RXR (Urquiza et al., 2000; Lengqvist et al., 2004) and PPAR (Chambrier et al., 2002) and have neuroprotective effects on the aged brain (Gamoh et al., 2001; Dyall et al., 2007). Retinoic acid receptors (RARs), RXRs, and PPARs are transcription factors involved in many cellular processes, such as learning and memory and their mRNA levels decrease with age (Chiang et al., 1998; de Urquiza et al., 2000; Chambrier et al., 2002; Lengqvist et al., 2004). Dyall et al. showed that RARα, RXRα, RXRβ, and PPARγ protein expression significantly decreases in the forebrain with ageing, and this was reversed by EPA and DHA supplementation (1.5:1 ratio). EPA and DHA supplementation could further restore the significant age-related decreases in RARα and RXRβ expression in CA1 and the dentate gyrus. DHA only supplementation also appeared to increase receptor expression in the prefrontal cortex, striatum, and hippocampus. Decreases in hippocampal Dcx expression were also partially alleviated (Dyall et al., 2010). Also my data show

for the first time that EPA and DHA prevent the Cortisol-induced decrease in Dcx positive cells in the Cortisol stress model in the HPC03A/07 cell line.

We are beginning to understand the different modes of action of EPA and DHA but we still don't understand why EPA is more efficacious than DHA in improving depressive symptoms especially as the concentration in the brain of DHA is a lot higher than the concentration of EPA. A possible explanation could be that EPA acts on endothelial cells and the cardiovascular system thereby increasing the blood flow to the brain which is associated with an increase in cognition (Tiehuis et al., 2008). My results support the positive effects of EPA and DHA on the survival, dividing cells, differentiation and maturation in a hippocampal progenitor cell line. By increasing the survival more dividing cells are available to go on to differentiate and mature. My results also confirm that EPA and DHA have different effects, as they may exert their effects through different pathways, which are yet to be elucidated. These results further the understanding on the beneficial effects of the Omega-3s EPA and DHA on the hippocampus, an area important for learning and memory and mood. They further add to the body of evidence suggesting Omega-3s as a potential treatment for depression and cognitive decline. However, the exact cellular mechanisms behind the effects of EPA and DHA still remained to be elucidated. Figure 3-27 shows an overview of the possible mechanisms involved in the effects of EPA and DHA in depression and cognition.

My results show for the first time the potential of dietary supplementation of Omega-3s in preventing a Cortisol induced decrease in survival and neurogenesis in human hippocampal progenitor cells. Although the cellular mechanisms behind their beneficial effects still remains to be elucidated, DHA

and EPA supplements have long been accepted to be beneficial in depressed patients. If Omega-3s were to be recommended as an additional or monothereapy for depression, or to improve learning and memory performance it will be important to bear in mind that not all individuals will respond to DHA and/or EPA in the same way which is also the case for antidepressants. Ongoing nutrigenetic research will be crucial in defining future advice regarding dietary and supplementary EPA and DHA. Further, supplementation is likely to be only effective in case of a prevailing deficit in EPA and DHA.

The next step is now to investigate the potential rescue abilities of EPA and DHA in the human hippocampal progenitor cell line. Experiments investigating the potential rescue properties of EPA and DHA using the Cortisol stress model in the HPC03A/07 cell line are currently ongoing in the Thuret lab.

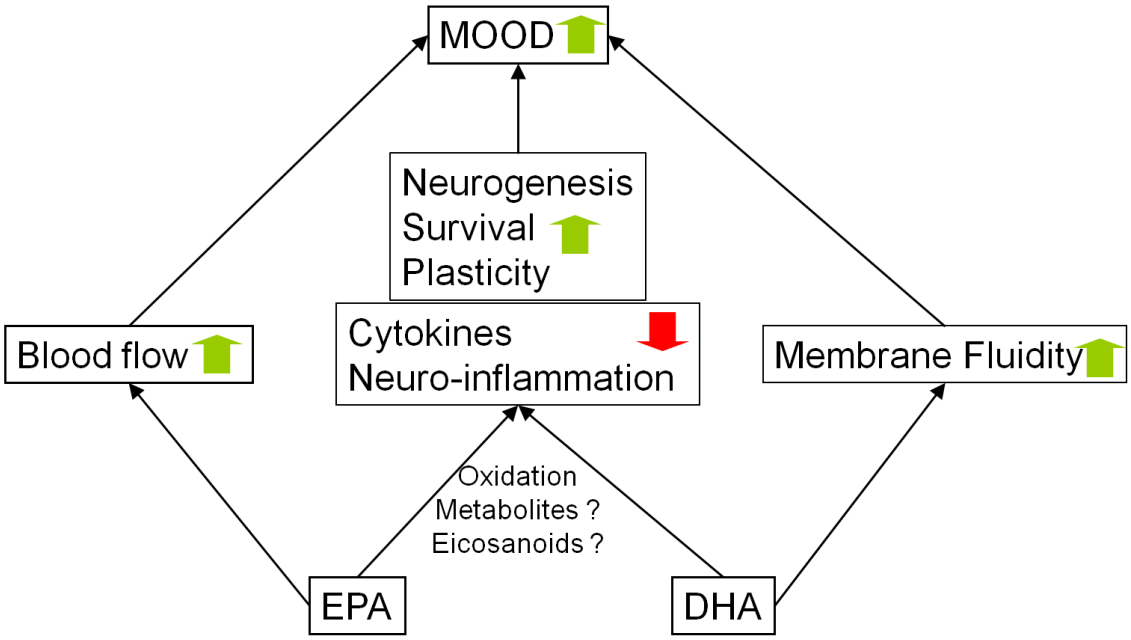


Figure 3-27 Overview of possible ways EPA and DHA affect mood

3.5.4 The stilbenoid Resveratrol increases the proportion of dividing cells and neuronal cells

My data show that RSVL increases the proportion of dividing cells and mature neuronal cells. The proportion of apoptotic cells is increased during differentiation. The effects of RSVL are dose dependent and the increasing effects on dividing cells and cell death are contained within a narrow range. When titrating the concentration for the highest rate of dividing cells, I did not investigate the percentage of dying cells. It might be that the concentration (1µM) I chose, lead to the greatest increase in proliferation but was still too high in respect to apoptosis. A lower concentration might still have given a significant increase in proliferation with lower (or absence of) apoptosis. Closer investigation of the effect of RSVL on cell death in the human embryonic progenitor cell line is required. It will further be necessary to examine which cell-type dies. These results are also in accordance with the *Klotho* over-expression data in 4.2, which also increases cell fate commitment and apoptosis in differentiating HPC03A/07 cells. RSVL impacts on proliferation and differentiation partly through *Klotho* activation shown in 5.4. RSVL is produced by plants as a phytoalexin to protect them from fungal and bacterial invasion. Phytoalexins are broad spectrum inhibitors that act as toxins to attacking organisms. This toxic potential explains its effects on increasing apoptosis in higher concentrations in HPC03A/07 cells. In short lived organisms such as fruit flies and nematodes, RSVL increases life span (Wood et al., 2004). In yeast RSVL feeding increases life span and also the activity of the enzyme Sirtuin2 (Sir2) that plays a key role in an organism's response to stresses, is associated with longevity and is also increased during caloric restriction (CR) (Lin et al., 2000; Howitz et al., 2003). Long term administration of RSVL induces similar

gene expression patterns to CR in mice and delays aging related deterioration, but does not extend life span when started in mid life (Pearson et al., 2008). RSVL acts on various targets that are associated with protection against lifestyle-related diseases, such as depression, diabetes and high blood pressure. Amongst those targets that are activated by 5µM RSVL are Sirtuin1, the mammalian homologue to Sir2, which is involved in cellular regulation, the nuclear transcription factors PPARα, δ and γ, COX1 and 2 that are key enzymes in prostaglandin biosynthesis and eNOS, the endothelial nitric oxide synthase that generates NO in blood vessels and regulates vascular tone *in vitro* in human cells (Nakata et al., 2012). However SIRT1 over-expression does not extend life span in healthy mice (Herranz et al., 2010), compared to its equivalent Sir2 in yeast (Howitz et al., 2003). Although RSVL might not extend lifespan in mammals it ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases in mice and consequently increases Nicotinamide adenine dinucleotide (NAD) and the activity of SIRT1 via AMPK (Park et al., 2012) See 1.3.2.1. Moreover, RSVL improves cognitive function in wild type mice by increasing production of insulin-like growth factor-I and enhancing angiogenesis and neurogenesis in the hippocampus (Harada et al., 2011). Also my data show an increase of mature neurons in the HPC03A/07 cell line after RSVL supplementation but no attenuating effect on apoptosis.

In human, a single dose of orally administered RSVL can modulate cerebral blood flow but has no effect on cognitive performance (Kennedy et al., 2010). A recently completed clinical trial by Haskell also found that RSVL supplementation increases cerebral blood flow in young healthy males but has no effect on cognition (http://clinicaltrials.gov/; Identifier: NCT01010009).

The pro-angiogenic effects of RSVL in brain endothelial cells are mediated via activation of PI3-K/MAPK/ERK signalling pathways and lead to improved blood pressure and blood flow (Simao et al., 2012). As stated previously, an improved cardiovascular system can be beneficial for brain functions, as increased blood flow in the brain is known to facilitate AHN (Nagahama et al., 2003; Ruitenberg et al., 2005). Based on its antioxidant, chemopreventive and anti-inflammatory action RSVL predominantly appears to be improving health in damaged systems, such as ischemic brain damage (Palmer et al., 2000), diabetes or cancer (Rivera et al., 2009). I therefore investigated the preventative effects of RSVL in the Cortisol stress model.

3.5.5 RSVL pre-treatment can prevent the negative effects of Cortisol on proliferation and differentiation, but not on apoptosis.

My data show that RSVL treatment of HPC03A/07 cells in the Cortisol stress model during proliferation increases the proportion of dividing cells most efficiently when present continuously, however not to the level of the RSVL only treatment. RSVL pre-treatment does not prevent apoptosis during both proliferation and differentiation. During differentiation pre-treatment only (R-- --CC) appears to be enough to increase dividing cells, neuroblasts and mature neuronal cells, to almost the vehicle level, however when cultures were treated also during proliferation once the Cortisol treatment was started (RR- -CC) the increase was even greater although not to the level of RSVL only but above the vehicle. Continuous RSVL treatment (RRR –CC) also during differentiation increased dividing and neuronal cells; however not as efficient as when cultures were treated during proliferation only. The increase in dividing cells, neuronal cells and apoptosis might be because RSVL treatment pushes cells rapidly into fate commitment leading to increased cell death. To my knowledge RSVL has

never before been studied in a human hippocampal cell line and never for its ability to prevent Cortisol induced stress. Further, very little is known todate about the effect of RSVL on neurogenesis. A study conducted recently by Girbovan et al. investigated the effect of RSVL pre-treatment on global ischemia in rats. They concluded that repeated RSVL administration leads to lasting protection against neuronal damage in the hippocampus but induces dose related alterations of behavior and memory (Girbovan et al., 2012). These data are similar to my results that indicate that RSVL can prevent the Cortisol induced effects on dividing cells, neuroblasts and mature neurons by increasing the proportion of dividing and neuronal cells by increasing proliferation and neurogenesis and not due to survival as the percentage of apoptotic cells is not decreased. RSVL can further attenuate the deleterious effects of focal cerebral ischemia/reperfusion-induced brain injury and function as a potential neuroprotective agent by decreasing apoptosis (Li et al., 2012). Contrary to this, my data show an increase in apoptosis during differentiation. RSVL is cytotoxic at higher concentrations and the concentration of 1µM used in my experiments, despite increasing the proportion of dividing cells most, might still elicit apoptotic effect and increase on cell death. It is well known that RSVL has cytotoxic effect on cancer cells and can reduce cell viability at high concentrations (Takahashi et al., 2009). However in non cancer cells RSVL (40mg/kg) improves hippocampal atrophy in chronic fatigue mice by enhancing neurogenesis and inhibiting apoptosis of granular cells (Moriya et al., 2011), which is similar to the neurogenic effect I observe, however the concentration I choose for this experiment does not decrease apoptosis. RSVL supplementation also attenuates obesity-associated peripheral and central inflammation and improves memory deficit in mice fed a high-fat diet (Jeon et al., 2012). RSVL shifts the

physiology of middle-aged mice on a high-calorie diet towards that of mice on a healthy diet and significantly increases their survival by impacting on molecules associated with longer lifespan, including increased insulin sensitivity, reduced IGF-I levels, increased AMPK and PGC-1alpha activity, increased mitochondrial number and improved motor function (Barger et al., 2008a). Moreover RSVL exerts anti-inflammatory effects in murine microglia and astrocytes by inhibiting different proinflammatory cytokines and key signalling molecules such as TNF-alpha, IL-6, iNOS/NO and NF-kappaB (Lu et al., 2010). RSVL exerts its protective effects against lifestyle-related diseases via various pathways involving: SIRT1, p53, NF-κB, PGC1α, eNOS, FOXO, PPARs, MAPK, AKT, PI_3K, cAMP, AMPK (Pirola and Frojdo, 2008; Nakata et al., 2012; Park et al., 2012). It appears that RSVL, as a pharmacological agent, has a wide target spectrum. The biological activities of RSVL may thus be dependent on its simultaneous activity on multiple molecular targets.

However it is becoming increasingly clear that RSVL has two faces. On one hand, it protects cells by potentiating a survival signal; on the other hand, it selectively kills cancer cells (Dudley et al., 2009; Takahashi et al., 2009; Edwards et al., 2010; Moriya et al., 2011; Aluyen et al., 2012). RSVL behaves as an antioxidant, yet it can induce redox signalling (Dudley et al., 2009). RSVL, similar to Cortisol, has a differential dose dependent effect. At moderate concentrations they are beneficial for survival, proliferation and neuronal differentiation and if the concentrations reach a certain threshold their effects become detrimental and can lead to cell death (3.2.1). It might be possible that RSVL has greater effects on survival, cell division and differentiation under preceding stress, such as elevated Cortisol levels, *Klotho* knock down (as discussed in 4.3) or diabetes, obesity and ischemia.

Although *in vitro* and *in vivo* animal experiments conclude that RSVL acts on pathways involved in cell cycle metabolism and improves symptoms of aging related diseases and cognitive function, it will be difficult to extrapolate these effects to human, as the concentrations used are difficult to achieve in human by diet alone and also RSVL is highly processed and therefore only blood vessels might get in contact with RSVL whereas the organs are left with its metabolites (Walle et al., 2004). This suggests that further research is needed into the metabolic effects of the RSVL metabolites have on cellular mechanisms, especially regarding neurogenesis.

Chapter 4 Expression of Klotho in mouse brain and its effect on proliferation and differentiation in vitro in HPC03A/07

In this chapter, I describe my findings related to Aim 2 the expression of Klotho in mouse brain and its impact on proliferation, differentiation and survival in HPC03A/07. *Klotho* is known as the 'ageing suppressor gene', due to the distinct symptoms of a recessive *Klotho* mutant mouse (kl/kl) resembling those in the course of the aging process in human (Kuro-o et al., 1997). The *Klotho* mutant phenotype includes a short lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis, emphysema, hypokinesis and gait disturbance. Histological analysis of the central nervous system in *kl/kl* mice revealed a decreased number of Purkinje cells, but there were no other age-related changes such as brain atrophy, senile plaques or amyloid deposits (Kuro-o et al., 1997). However, they did not look at neurogenesis in kl/kl mice. In turn, in a mouse model where *Klotho* is over expressed the lifespan of this mouse is extended by up to 30%. Further, Insulin and IGF1 resistance increases by suppression of tyrosine phosphorylation of the insulin and IGF1 receptors (Kurosu et al., 2005). The effect of *Klotho* over-expression on the brain has not been analysed.

As described in 1.4. Klotho is a single pass transmembrane protein that consists of two external β-glucosidase-like domains (hKL1 and hKL2), a transmembrane domain and very short cytoplasmic domain. The membrane domain facilitates the high affinity binding of FGF23 to the FGF receptor and is therefore involved in the endocrine regulation of phosphate homeostasis as part of a bone-kidney-parathyroid axis (Hu et al., 2010). The external domain can be shed by alternative splicing of the mRNA and proteolytic cleavage of the protein in the area of Exon 3 leading to a secreted form of *Klotho* (Chen et al., 2007; Bloch et al., 2009; Wang and Sun, 2009). The secreted Klotho can be found in blood, urine and Cerebrospinal fluid (CSF) and explains the pleiotropic, cell

non-autonomous effects of this gene (Kuro-o et al., 1997). Klotho is primarily expressed in the Kidney. However, German et al. have just described the expression of transmenbrane Klotho in the mouse brain. They have found transmembrane Klotho to be mostly expressed in the choroid plexus off the SVZ and in cerebellar Purkinje cells. It is expressed in the plasma membrane as well as near the nuclear membrane (German et al., 2012).

Recently we observed that *Klotho* expression is increased in rodents upon intermittent fasting specifically in the hippocampus. Moreover, this up regulation of *Klotho* expression was associated with increased AHN and improved retention memory in these animals (Data has not yet been published, but was described in this thesis 1.3.4. Altogether these data suggest a potential role for *Klotho* in regulating adult hippocampal neurogenesis.

First I wanted to investigate what cell types in the mouse hippocampus express Klotho. Second, I will show that Klotho expression increases in the hippocampus of mice maintained on an intermittent fasting diet. Third, to be able to work under controlled conditions I went on to investigate the effects of *Klotho* over-expression and *Klotho* knock down *in vitro* on HPC03A/07 proliferation and differentiation.

4.1 Klotho protein is expressed in the dentate gyrus of the mouse brain

To assess the co-expression of Klotho in the hippocampus in the brain of 3 months old mice, PFA fixed wild type mouse brain sections were immunohistochemically stained for Klotho and co-labelled for SOX2, NeuN or GFAP. Sections were further co-labelled for Nestin, Ki67 and Dcx. I have than qualitatively assed which cell types in the hippocampus express Klotho protein. In the hippocampus, Klotho is predominantly expressed in the CA 1,2 and 3 and the dentate gyrus. Figure 4-1 shows that Klotho is co-expressed in NEUN and SOX2 positive cells, but not in GFAP positive cells. Further data showed that Klotho is not expressed in Nestin, Ki67 or Dcx positive cells (data not shown). This indicates that Klotho is expressed in mature neurons and putative stem cells, but not in astrocyts.

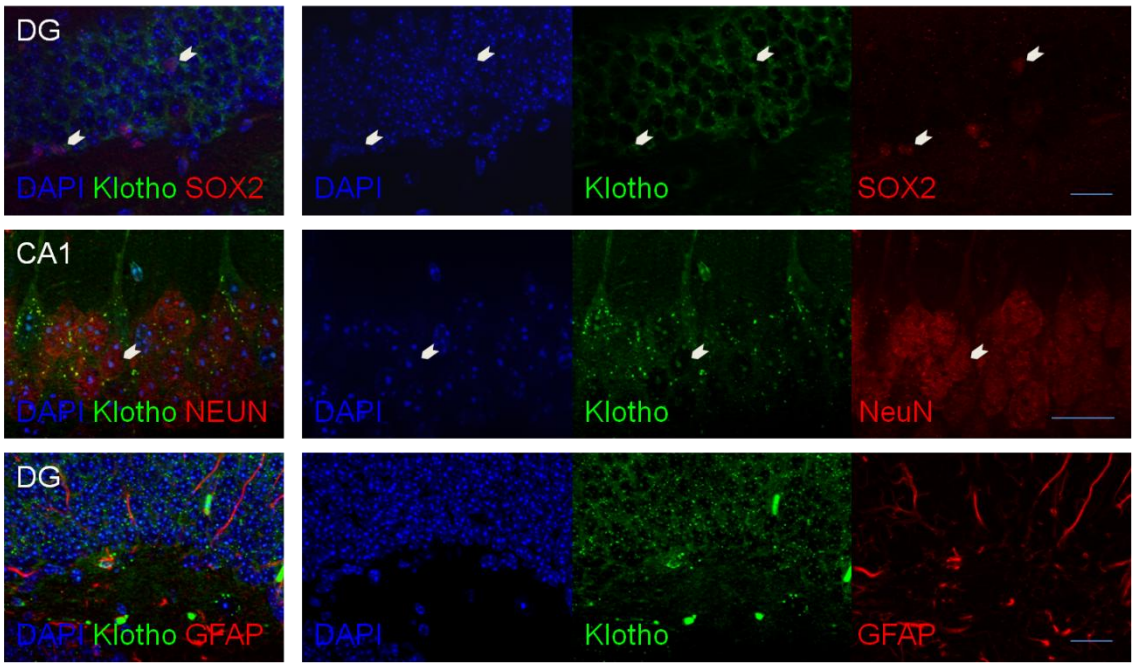


Figure 4-1 Klotho expression in wild type mouse brain
In wild type mouse brain in the hippocampus Klotho co-localises with NEUN and SOX2 but not with GFAP. Arrowheads point at Klotho co-expressing cells. (Scalebar: 20µm)

4.1.1 Klotho expression in the hippocampus of adult mice upon intermittent fasting

As described in Section 1.3.4 *Klotho* mRNA expression was increased in the hippocampus of mice on an intermittent fasting diet. To investigate the changes in Klotho protein expression in the hippocampus of these mice, I then went on to immunohistochemically assess changes in Klotho protein expression in the hippocampus of the intermittent fasting (IF) mouse model as described in Section 2.5.2. It was assumed that the increased AHN was due solely to reduced calorie intake, however unpublished pilot work by Sandrine Thuret's lab has shown that increased AHN in dietary restriction paradigms is not only dependent of calorie intake but also depends on meal frequency in the form of intermittent fasting (See 1.3.4). In this IF model, C56Bl/6 mice restrict their caloric intake automatically by 10% over two days. Under calorie restriction (CR) the daily measured calorie intake is restricted to 90% of AL calorie intake. Briefly, three groups of animals were kept for three months under either ad libitum (AL), calorie restriction (CR) or IF conditions. Stereological analysis was conducted as described in 2.6.1.2 and threshold analysis was conducted as described in Section 2.6.1.3.

Klotho positive cells were found in the hippocampus (images in Figure 4-2) with some Klotho expressing cells with processes observed inside the DG and inside the blade of the DG (Figure 4-2 CR and IF, arrow). Stereological quantification of Klotho positive cells in the DG of the hippocampus did not show significant differences in the IF or the CR group compared to the AL fed mice, see Figure 4-2.

Nonetheless the Q-PCR results for *Klotho* expression showed a twofold increase in *Klotho* mRNA expression in the IF group compared to the AL and CR groups. Moreover, while blinded to the code, I also noted a difference in intensity in Klotho expressing cells in some of the brains. Consequently I decided to conduct threshold analysis that showed a significant increase of Klotho protein in the IF group compared to the AL control group by 335.5±72.64, $p<0.01$ (Figure 4-3) but not in the CR group, suggesting that the number of Klotho expressing cells does not increase, but that upon IF the existing Klotho positive cells produce more protein, see Figure 4-3 images.

Altogether, the results described above showed that Klotho is co-expressed in neurons and dividing cells in the dentate gyrus in the mouse brain and that Klotho expression is increased upon IF diet which is associated with increased neurogenesis in the dentate gyrus and improved memory (see 1.3.4). Next, I decided to continue to work *in vitro* in the human hippocampal HPC03A/07 cell line to be able to study the effect of Klotho on human proliferation and neurogenesis and to investigate the molecular mechanisms behind it under controlled conditions. Therefore, I first analysed the effects of *Klotho* over-expression in a conditionally *Klotho* over expressing subclone of the HPC03A/07 cells (KLover) to investigate the effect of the humoral active secreted form of Klotho on proliferation, differentiation and survival. Based on the results in the IF *in vivo* model, I hypothesised that an increase in *Klotho* expression *in vitro* should entail an increase in neurogenesis. Second I knocked down *Klotho* secreted and membrane expression in HPC03A/07 cells using siRNA to investigate whether *Klotho* is required for proliferation, differentiation and survival.

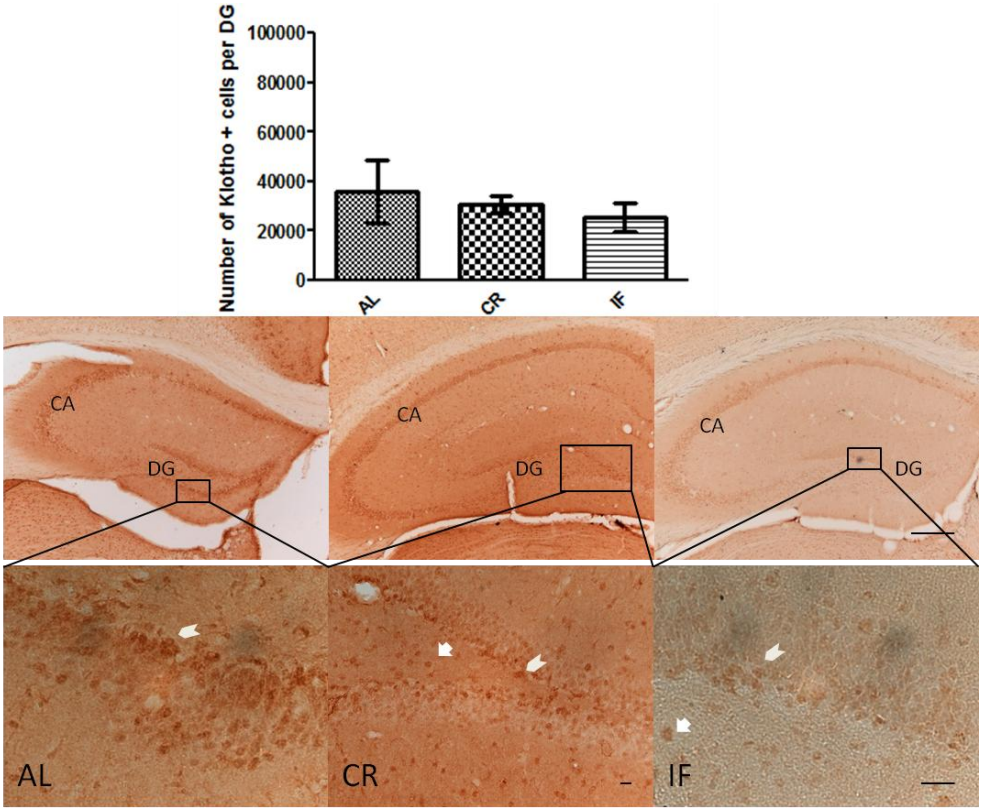


Figure 4-2 Stereological analysis of the number of Klotho expressing cells in the DG of ad libitum (AL), caloric restriction (CR), Intermittent fasting (IF) fed mice.
Histogram of stereological quantification in the Dentate Gyrus (DG): There is no difference between the groups in the number of Klotho expressing cells. Top row images show the whole hippocampus, bottom row images show the DG at higher magnification of coronal sections. Arrow head: Klotho positive cells inside DG; Arrow: Klotho positive cells inside the blade of the DG (Scalebar: top: 100µm; bottom: 20µm; AL n=3, CR n=5, IF n=5, One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM)).

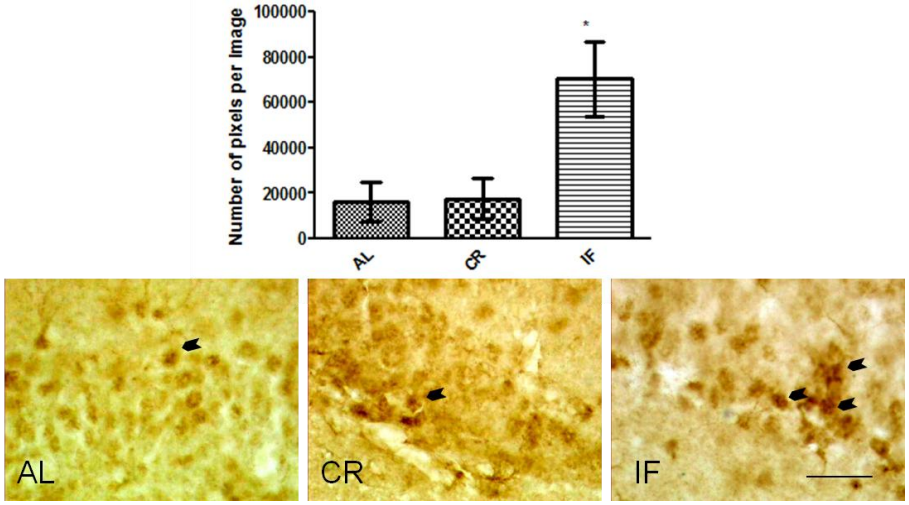


Figure 4-3 Klotho protein threshold analysis in the dentate gyrus for Klotho of ad libitum (AL), caloric restriction (CR), Intermittent fasting (IF) fed mice.
Klotho protein threshold analysis showed a significant increase in the intensity of the Klotho staining in Klotho positve cells in the IF group compared to the AL and CR group. Arrows indicate Klotho positive cells with higher intensity in the IF group compared to AL and CR group. (Scalebar: 20µm, One-Way ANOVA with Newman-Keuls post hoc test Error bars are the Standard Error of the Mean (SEM), *p<0.05)

4.2 Klotho over expression in Klover cells *in vitro*

To assess the effect of the secreted form of the gene *Klotho* on the proliferation and differentiation of HPC0A07/03A cells, these cells were genetically engineered to conditionally over-express this form of *Klotho* using the Lenti-X™ Tet-On® Advanced Inducible Expression System (Clonetech). Cells were selected for successful transfection by antibiotic selection, see 2.1.8. Gene expression is activated in this system using the tetracycline Doxycycline, leading to a stable *Klotho* over-expression. After the over-expression of *Klotho* was started, cultures were maintained for either 3 days under proliferation conditions or for 7 days under differentiation conditions in order to assess the effect of Klotho on proliferation, differentiation and survival.

4.2.1 Validation of Klotho over expression in Klover cells using Q-PCR and Western Blot

To confirm the over-expression of *Klotho* in Klover cells at the mRNA level I used Q-PCR (SYBR green). These Q-PCR values were normalised against the geometric mean of the three normalisers UBC, HPRT1 and β-actin that were chosen as their expression remains unchanged when *Klotho* is over-expressed. Figure 4-4 shows a comparison of *Klotho* mRNA levels from Klover cells in which *Klotho* over-expression was not switched on (OFF) and the ON group in which *Klotho* was over-expressed in proliferating (Figure 4-4a) and differentiating (Figure 4-4b.) Klover cells. In ON proliferating Klover cells *Klotho* mRNA expression was non-significantly increased. In differentiating Klover cells, *Klotho* mRNA expression was significantly increased to 34.3±10.3%, $p<0.05$ in the ON group compared to the OFF control. *Klotho* over-expression is a lot higher during differentiation than during proliferation possibly because 7

days differentiation allows additional time for the expression of *Klotho* compared to only 3 days proliferation. However, *Klotho* over-expression during proliferation shows a significant increase in protein (Figure 4-5a.) despite the relatively low increase in mRNA levels indicating that even a minor increase in mRNA is sufficient for protein level to be significantly increased.

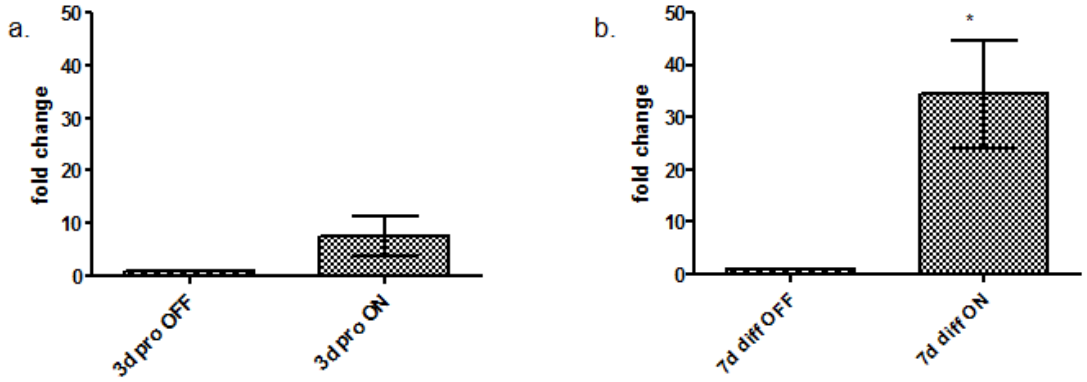


Figure 4-4 Changes in mRNA levels upon *Klotho* over-expression in proliferating and differentiating HPC03A/07

Klotho expression is significantly increased in Klover cells after *Klotho* over-expression was initiated in differentiating cells (b) but not proliferating cells (a). OFF: Klover cells where *Klotho* over-expression is not switched on; ON: Klotho overexpression is switched on. (Student's *t*-test, Error bars are the Standard Error of the Mean (SEM);*$p<0.05$)

To confirm the successful over-expression of the secreted form of Klotho protein, western blot analysis was carried out on both whole cell lysates of Klover cells and on supernatant collected from these cells after 3 days proliferation and 7 days differentiation in culture. Proteins were visualised using the Odyssey detection system. The relative density of the Western blot bands was analysed using ImageJ software. The intensity of the band was plotted as relative percentage of Klotho protein against a blank. In proliferating ON Klover cultures (Figure 4-5a) the secreted form of Klotho was observed to be increased by 22.8±0.9%, $p<0.0001$ in the supernatant and by 27.2±0.002%, $p<0.0001$ in the whole cell lysate. In differentiating ON Klover cultures (Figure 4-5b) the secreted form of Klotho was increased by 25.9±2.9%, $p<0.001$ in the supernatant and by 21.5±3.5%, $p<0.01$ in the whole cell lysate. This indicates a successful over-expression of Klotho protein during proliferation and differentiation in whole cell lysate and supernatant.

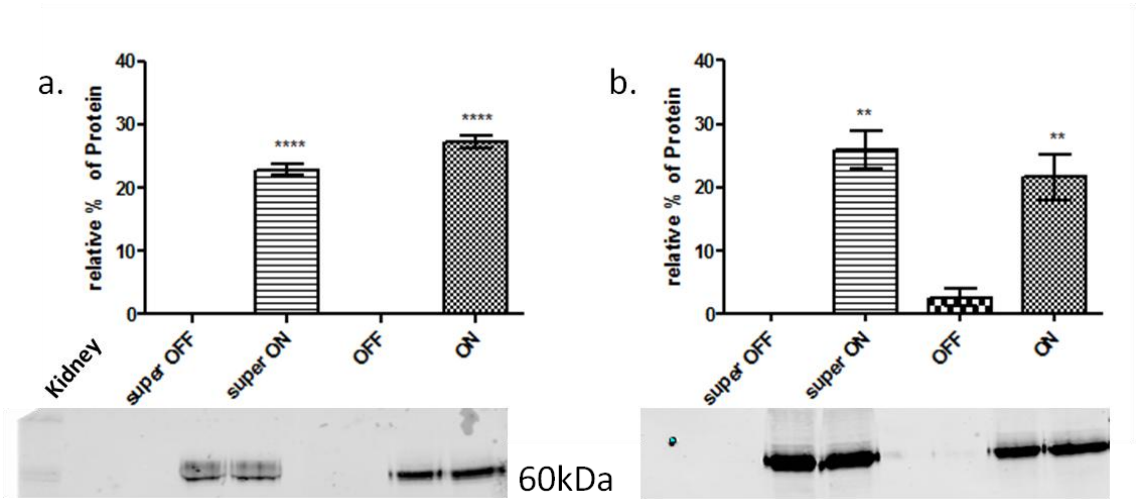


Figure 4-5 Klotho protein levels in whole cell lysates and supernatant from proliferating (a) and differentiating (b) Klover cells

Klotho protein was increased in the supernatant and the whole cell lysate in Klover ON cells compared to Klover OFF cells. Abbreviations: Super: supernatant; OFF: Klotho over-expression is not switched on; ON: Klotho overexpression is switched on. (Student's *t*-test, Error bars are the Standard Error of the Mean (SEM); **p<0.01, ****p<0.00001)

4.2.2 Immuncytochemistry upon *Klotho* over expression in HPC03A/07

After I confirmed the successful over-expression of the secreted form of Klotho in Klover cells in the presence of Doxycycline (Dox) on mRNA and protein level I now wanted to investigate the effect *Klotho* over-expression on proliferation, cell fate commitment and survival. Therefore, cultures were grown either under proliferative (3 days) or differentiative conditions (7 days) in the presence and absence of Dox before they were fixed with 4% PFA. Cultures were stained immunocytochemically with markers that identify proliferating (KI67) and apoptotic cells (activated Caspase-3) or progenitor cells (SOX2 and Nestin). Under differentiation conditions cells were also stained with markers identifying neuroblasts (Dcx), mature neurons (MAP2) and astrocytes (S100b). Numbers are presented as percentage change of Klover ON compared to Klover OFF cells.

4.2.2.1 SOX2 is increasingly expressed in the cytoplasm of proliferating Klover cells

As expected after 3 days of proliferation Klotho expression in Klover ON cells was significantly increased compared to Klover OFF cells (1245±27.6%, $p<0.0001$) (Figure 4-6b) with 45.2±0.9% of total cells expressing Klotho (Figure 4-6a). Although the percentage of cells expressing nuclear SOX2 (total 99.6±0.1%, Figure 4-6c) was significantly decreased (-0.4±0.1%, $p<0.0001$) compared to Klover OFF cells (Figure 4-6d), the percentage of cells expressing SOX2 in the cytoplasm was significantly increased by 146.8±7.8%, $p<0.0001$ (Figure 4-6f) with a total of 6.8±0.8% positive cells, Figure 4-6f. SOX2, a nuclear transcription factor, is essential to maintain self-renewal of undifferentiated embryonic stem cells. SOX2 proteins are regulated in part by nuclear import and export (Li et al., 2007). Excluding a transcription factor such as SOX2 from

the nucleus blocks its ability to bind to target DNA sequences and control target gene expression, thereby prohibiting it to drive the transcription of its target genes and in the case of SOX2 eventually rendering the cells incapable of self-renewal and pluripotency. The percentage of cells expressing Nestin was also decreased by -0.59±0.1%, $p<0.0001$ in Klover ON cells (Figure 4-6h) with a total of 99.4±0.1%, Figure 4-6g Nestin positive cells. Ki67 expression showed no significant changes with a total of 47.4±1.9% Ki67 positive cells in Klover ON cells (Figure 4-7b). Activated Caspase-3 showed a not significant increase compared to Klover OFF cells with a total of 4.3±0.2% positive cells in Klover ON cells (Figure 4-7f).

These data suggest that after initial increased proliferation, Klover ON cells might cease self-renewal due to *Klotho* over-expression.

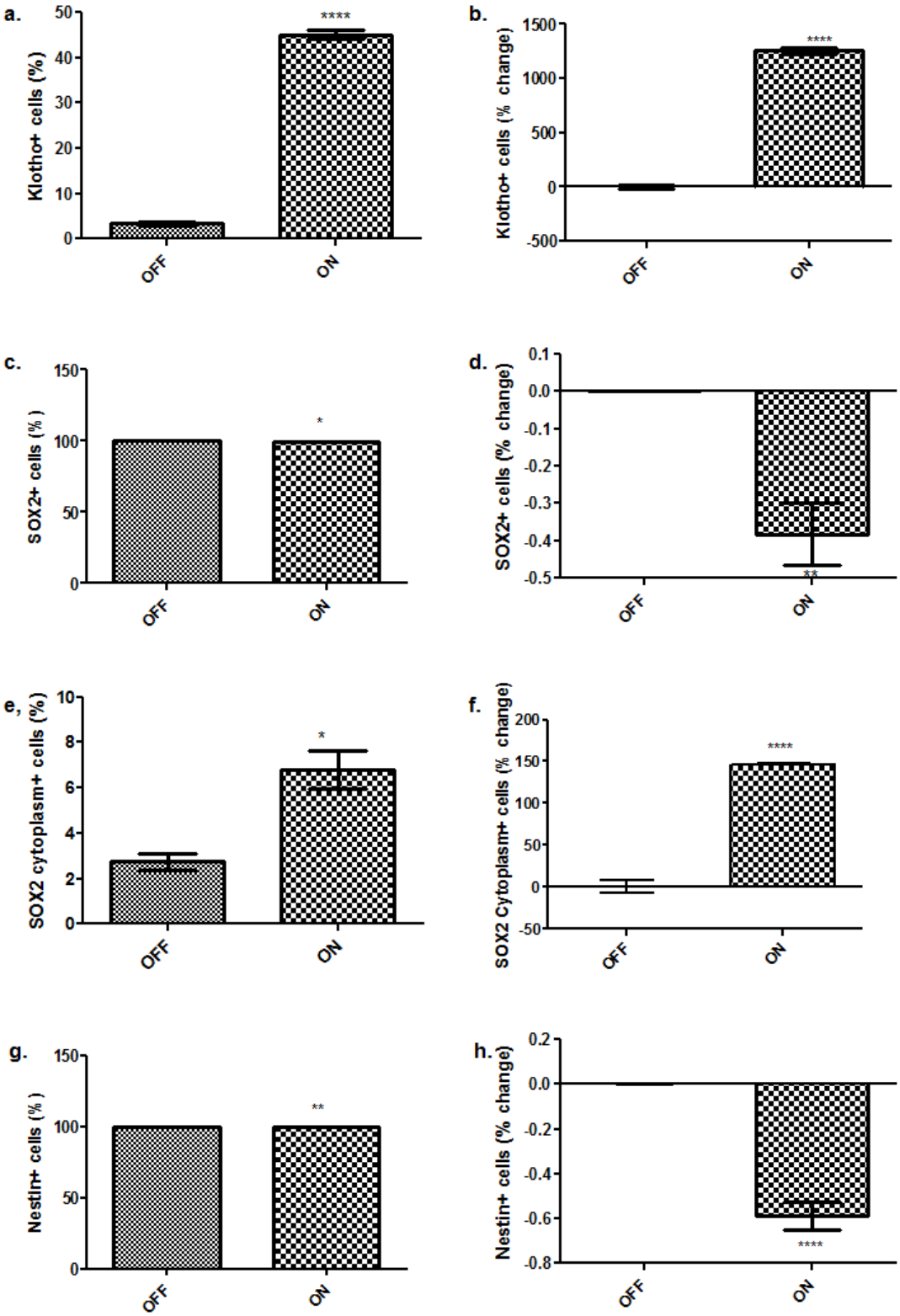


Figure 4-6 Klotho, SOX2, SOX2 cytoplasm and Nestin expression in proliferating Klover cells

a, d, e show the percentage of positive cells relative to the total number of cells counted. b, d, f show the percentage change of the marker in Klover ON cells compared to Klover OFF cells. a) and b) Klotho expressing cells. c) and d) SOX2 expressing cells. e) and f) SOX2 cytoplasm expressing cells. g) and h) Nestin expressing cells. Abbreviationss: OFF: Klotho over-expression is not switched on; ON: Klover cell line overexpressing the secreted form of Klotho. (Student's *t*-test, Error bars are the Standard Error of the Mean (SEM); ****P<0.0001)

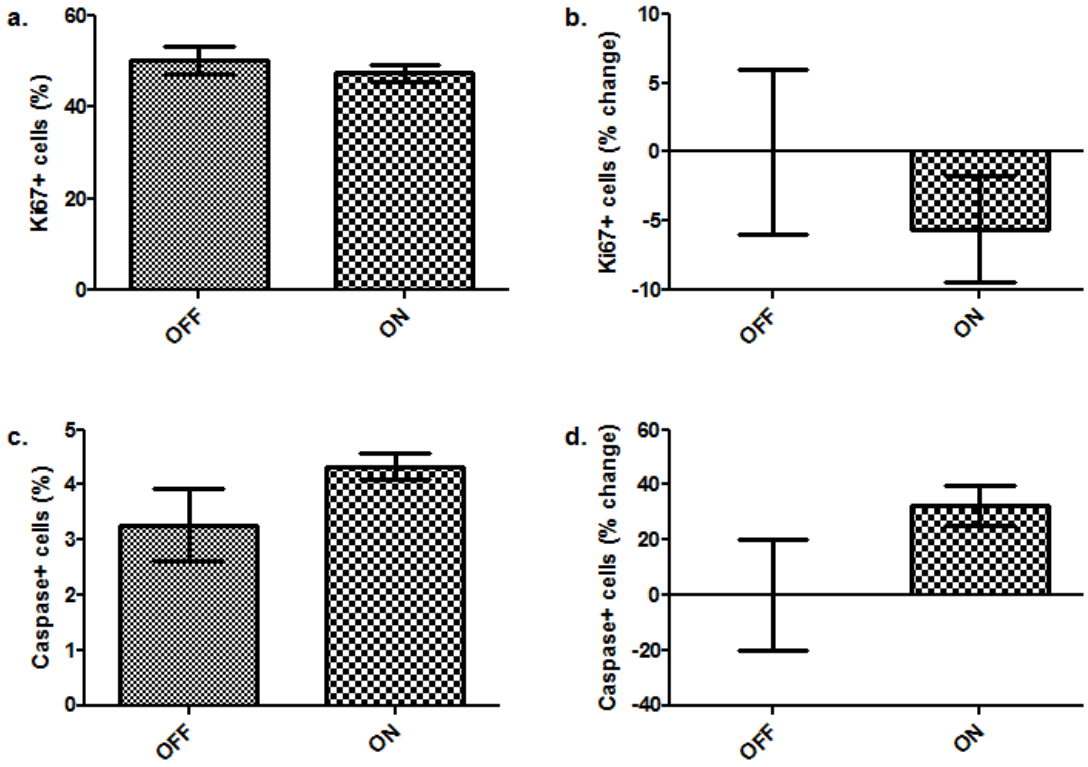


Figure 4-7 Ki67 and activated Caspase-3 expression in proliferating Klover cells a and c show the percentage of positive cells relative to the total number of cells counted. b and d show the percentage change of the marker in Klover ON cells compared to Klover OFF cells. a) and b) Ki67 expressing cells of total cells. c) and d) activated Caspase-3 expressing cellsof total cells. Abbreviationss: OFF: Klotho over-expression is not switched on; ON: Klover cell line overexpressing Klotho secreted. (Student's *t*-test, Error bars are the Standard Error of the Mean (SEM); *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001)

4.2.2.2 Klotho over-expression increased cell fate commitment and apoptosis in differentiating Klover ON cells

After investigating the effect of *Klotho* over-expression on proliferating HPC03A/07 cells I also wanted to examine its effects on differentiation. After 7 days of differentiation cultures were treated as described in Section 4.2.2. As expected the percentage of cells expressing Klotho was significantly increased upon *Klotho* over-expression by 378.6±40.5%, $p<0.001$ in Klover ON cells compared to Klover OFF cells to a total of 37.9±2.7% Klotho positive cells (Figure 4-8). The percentage of Dcx expressing cells was significantly increased to a total of 12.7±1.2% in Klover ON cells (percentage change from Klover OFF: 127.8±21.9%, $p<0.01$) (Figure 4-9a,b,c) and the proportion of MAP2 positive cells was significantly increased to 28.7±9.8% (percentage change from Klover OFF: 245.3±80.1%, $p<0.05$) (Figure 4-9d,e,f). S100b, an astrocyte marker, was significantly increased by 67.9±14.4%, $p<0.05$ to a total of 22.8±1.9% (Figure 4-10a,b,c). Activated Caspase-3 also showed a significant increase by 143.3±14.9%, $p<0.01$ upon *Klotho* over-expression to a total of 7.8±0.5% (Figure 4-12). A total of 31.2±4.4% cells expressed Ki67 in Klover ON cells leading to a non-significant change from Klover OFF cells (Figure 4-11). Nuclear SOX2 expression was not changed (total: 96.4±1.6%), whereas the percentage of cells that express SOX2 in the cytoplasm is significantly increased by 97.45±12.42%, $p<0.05$ to a total of 2.2±0.1%. Nestin expression is significantly decreased by -4.2±1.1%, $p<0.05$ (Figure 4-13) to a total of 94.9±1.0%.

The proportion of neuronal cells increases as do astrocytic cells, but there is no or very little change in dividing cells and cells labelled for SOX and Nestin. However, these data together with the increase in SOX2 cytoplasm positive

cells suggest that *Klotho* over-expression pushes the cultures into cell fate commitment.

When analysing the S100b data I noticed that S100b is not expressed in Klotho positive cells in the Klover OFF group but is expressed in Klotho positive cells in the Klover ON group. Therefore I went on to examine the proportion of Klotho positive cells co labelling with S100b in the Klover ON group. I found that 29.3±6.7% of Klotho positive cells co-express S100b in the Klover ON group compared to none in the OFF control (Figure 4-10d,e). I also analysed the proportion of Ki67, Dcx, MAP2 and activated Caspase-3 positive cells in the Klotho positive population; however there were no significant changes compare to their proportion in the Klotho population in the OFF group. See Figure 4-9-Figure 4-12 for pictures, graphs are not shown as no change cna be detected.

To sum up in the total cell population Klotho, Dcx, MAP2, S100b, activated Caspase-3 and SOX2 in cytoplasm are significantly increased, with S100b being only expressed in Klotho positive cells in the Klover ON group. This correlates with the staining in mouse brain where GFAP (another astrocytic marker) is not co-expressed in Klotho positive cells suggesting that the effect Klotho has on astrocytes is elicited by the secreted form as Klotho is not normally expressed in astrocytes. It would be interesting to examine the brains of *Klotho* mutant mice and of mice over-expressing Klotho to analyse which cell types co-express Klotho. Further investigation is also needed on which cell types are dying when *Klotho* is over-expressed by co-labelling cells for activated Caspase-3 and Dcx, MAP2, S100b and Ki67. A possible explanation for the increased cell death could be that over expressing the secreted form of *Klotho*

accelerates fate commitment to such an extent, which in turn could be leading to increased cell death.

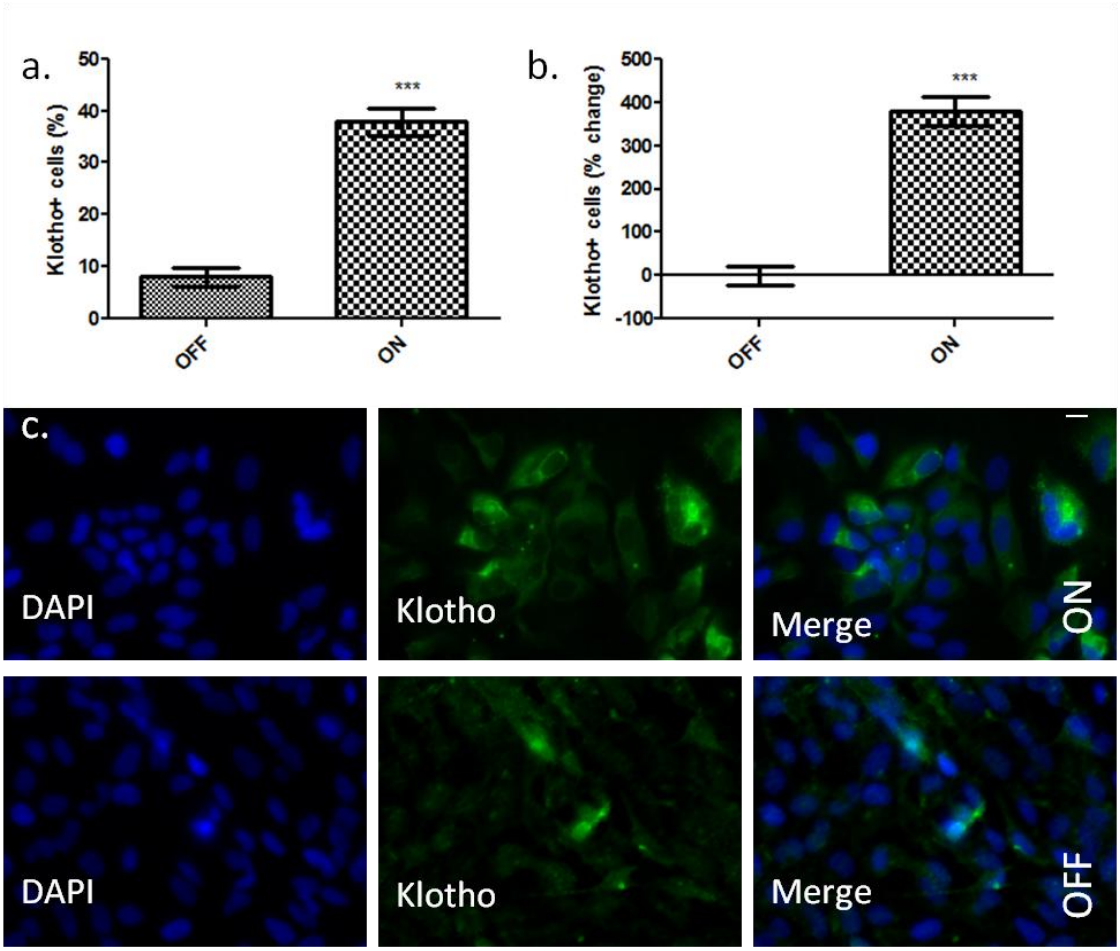


Figure 4-8 Klotho expression in differentiating Klover cells

a) Comparison of the absolute percentage of Klotho positive cells in Klover OFF and Klover ON cells. b) Percentage change of Klotho expression compared to the OFF control c) Images in the top row show Klover ON cultures, bottom row shows Klover OFF. OFF: Klotho over-expression is not switched on; ON: Klover cell line overexpressing Klotho secreted. (Scalebar: 20µm; Student's *t*-test, Error bars are the Standard Error of the Mean (SEM); ***p<0.001)

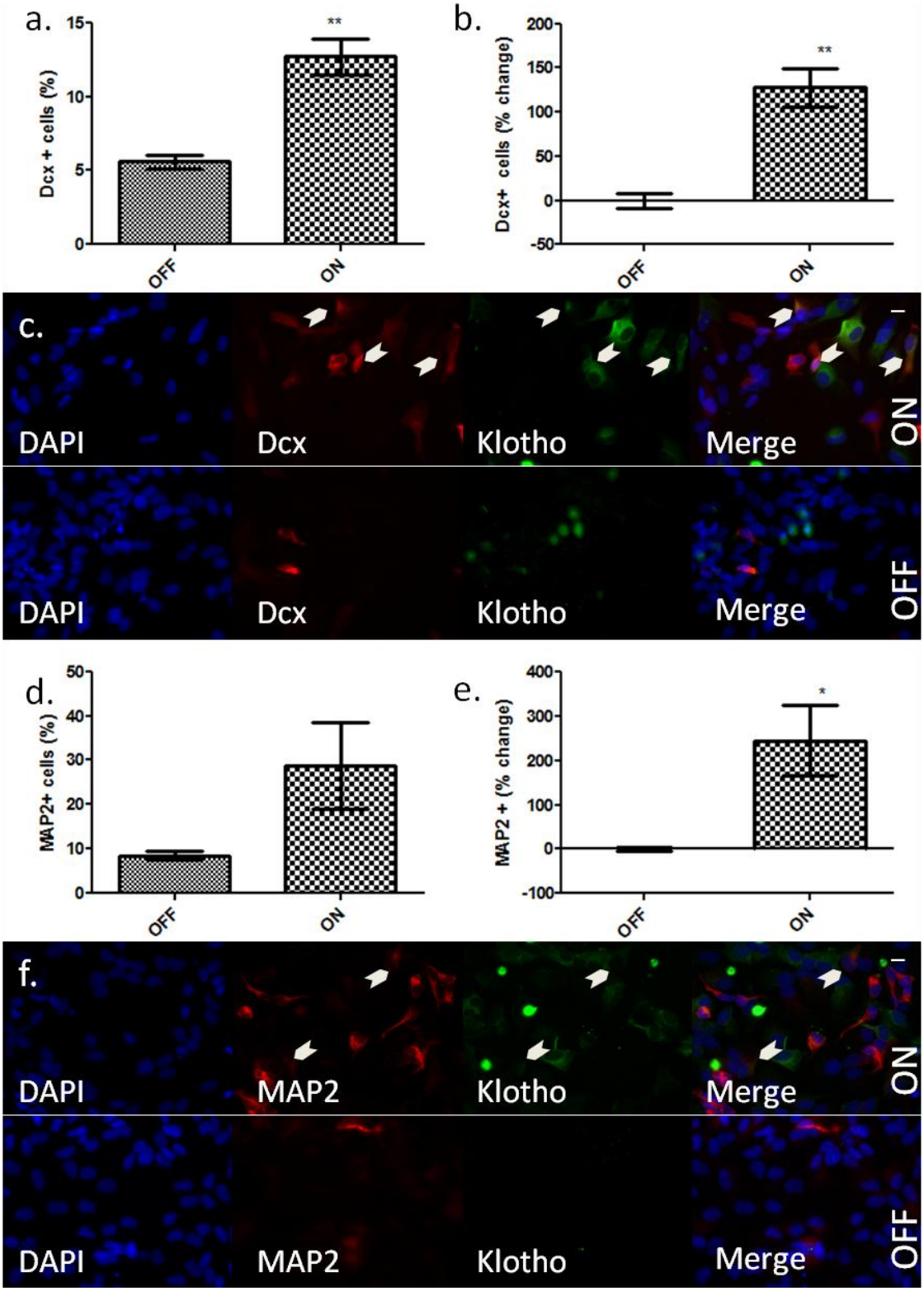


Figure 4-9 Dcx and MAP2 expression in differentiating Klover cells

a) Comparison of the absolute percentage of Dcx positive cells in Klover OFF and Klover ON cells. b) Percentage change of Dcx expression compared to the OFF control d) Comparison of the absolute percentage of MAP2 positive cells in Klover OFF and Klover ON cells. e) Percentage change of MAP2 expression compared to the control c) and f) Images in the top row show Klover ON cultures, bottom row shows Klover OFF. Arrows indicate double labelled cellsAbbreviations: OFF: Klotho over-expression is not switched on; ON: Klover cell line overexpressing Klotho secreted. (Scalebar: 20µm; Student's *t*-test, Error bars are the Standard Error of the Mean (SEM); *p<0.05, **p<0.01)

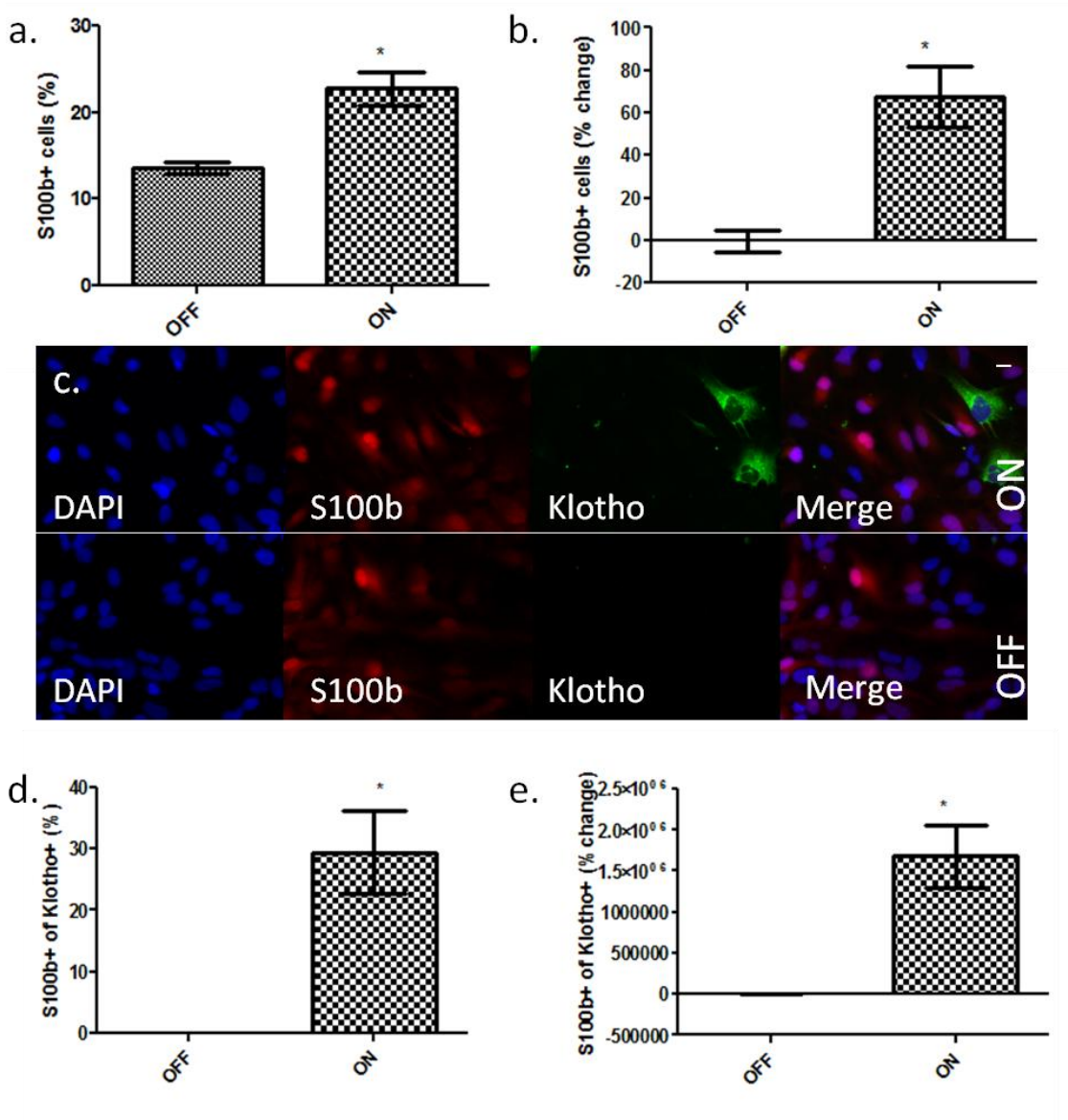


Figure 4-10 S100b expression in differentiating Klover cells

a) Comparison of the absolute percentage of S100b positive cells in Klover OFF and Klover ON cells. b) Percentage change of S100b expression compared to the OFF control . c) Images in the top row show Klover ON cultures, bottom row shows Klover OFF. d) Comparision of the percentage of S100b positive cells in the population of Klotho expressing cells e) Percentage of change of S100b and Klotho expressing cells compared to the Klotho positive cells. Abbreviations: OFF: Klotho over-expression is not switched on ;ON: Klover cell line overexpressing Klotho secreted. (Scalebar: 20µm; Student's *t*-test, Error bars are the Standard Error of the Mean (SEM), *p<0.05)

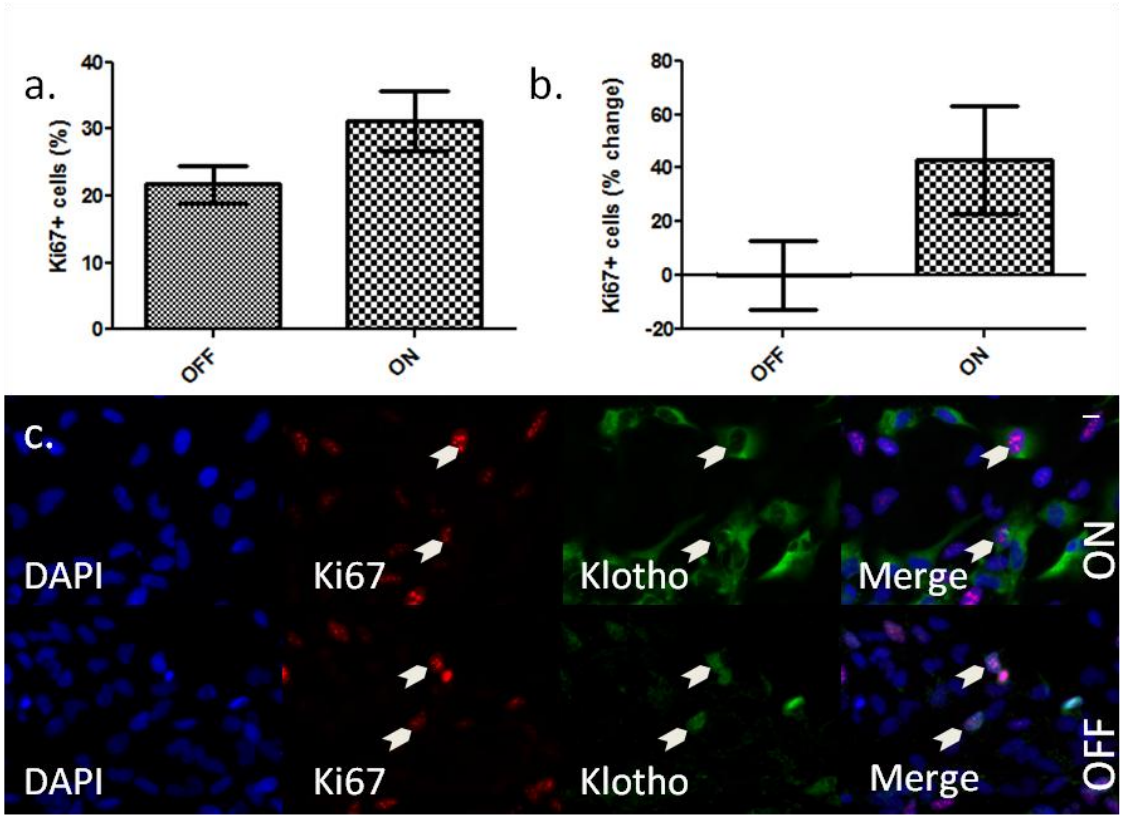


Figure 4-11 Ki67 expression in differentiating Klover cells

a) Comparison of the absolute percentage of Ki67 positive cells in Klover OFF and Klover ON cells b) Percentage change of Ki67 expression compared to the OFF control c) Images in the top row show Klover ON cultures, bottom row shows Klover OFF. Arrows indicate double labelled cells. Abbreviationss: OFF: Klotho over-expression is not switched on ; ON: Klover cell line overexpressing Klotho secreted. (Scalebar: 20µm; Student's *t*-test, Error bars are the Standard Error of the Mean (SEM))

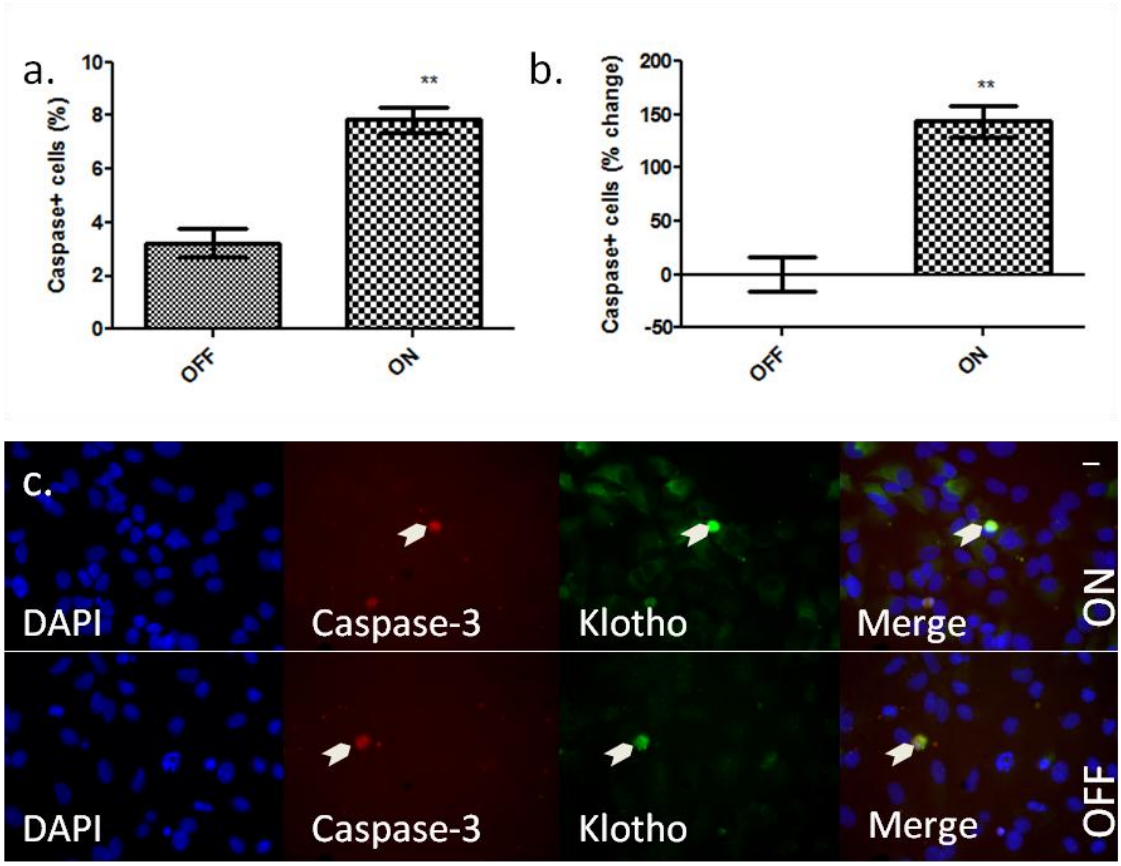


Figure 4-12 Activated Caspase-3 expression in differentiating Klover cells
a) Comparison of the absolute percentage of activated Caspase-3 positive cells in Klover OFF and Klover ON cells b) Percentage change of activated Caspase-3 expression compared to the OFF control c) Images in the top row show Klover ON cultures, bottom row shows Klover OFF. Arrows indicate double labelled cells. Abbreviationss: OFF: Klotho over-expression is not switched on; ON: Klover cell line overexpressing Klotho secreted. (Scalebar: 20µm; Student's *t*-test, Error bars are the Standard Error of the Mean (SEM); **p<0.01)

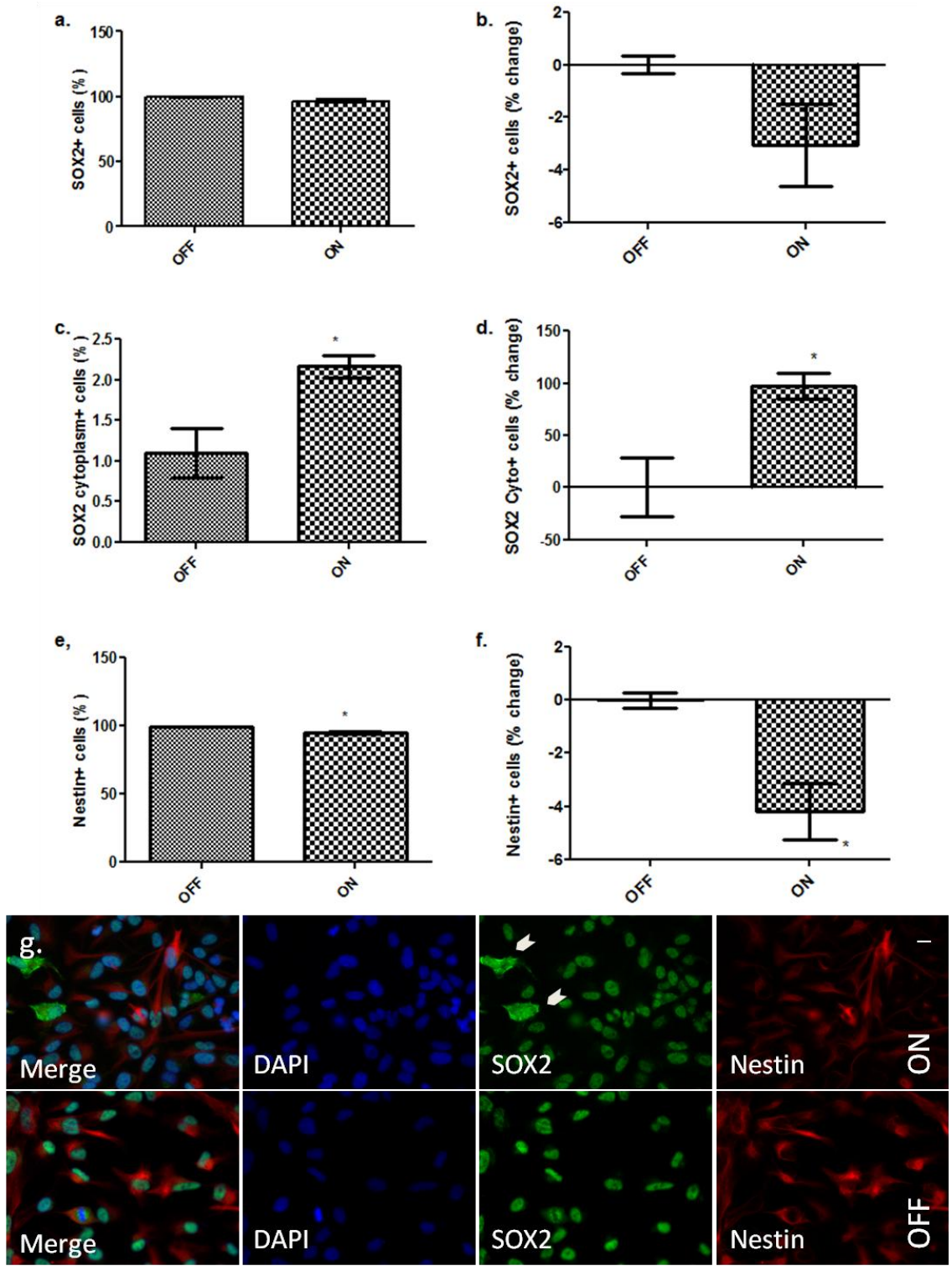


Figure 4-13 SOX2 and Nestin expression in differentiating Klover cells
a) and b) The percentage of SOX2 positive cells observed after *Klotho* over-expression shows no change. c) and d) SOX2 expression in the cytoplasm is significantly increased in ON cells compared to OFF cells e) and f) Nestin expression is significantly decreased in the On cells compared to the OFF cells. g.) Images show in the top row Klover ON cultures, the bottom row shows Klover OFF cultures The arrows indicate cells wher SOX2 is expressed in the cytoplasm. OFF: Klotho over-expression is not switched on; ON: Klover cell line overexpressing Klotho secreted. (Scalebar: 20µm; Student's *t*-test, Error bars are the Standard Error of the Mean (SEM); *p<0.05)

4.3 *Klotho* knock down *in vitro* in HPC03A/07 cells

To assess the effects of suppressing *Klotho* expression on HPC03A/07 cells, cultures were transfected using the N-TER nanoparticle system with three different siRNA and a mix of all three to evaluate the effect of diminishing *Klotho* expression on proliferation and differentiation. HPCOA07/03 cells were transfected as described in 2.1.7. 24h after seeding and for the proliferation assay maintained under proliferating conditions for 3 days. For the differentiation assay, differentiation was started 24h after siRNA transfection. Cultures were maintained under differentiation conditions for either 3 or 7 days as indicated in Figure 4-14 and were then fixed with 4%PFA for immunocytochemistry. Cultures were stained with markers that identify proliferating (KI67) and apoptotic cells (activated Caspase-3) or progenitor cells (SOX2 and Nestin). Under differentiation conditions cells were also stained with markers identifying neuroblasts (Dcx), mature neurons (MAP2) and astrocytes (S100b). Numbers are presented as percentage change of *Klotho* knock down compared to control siRNA transfected cells.

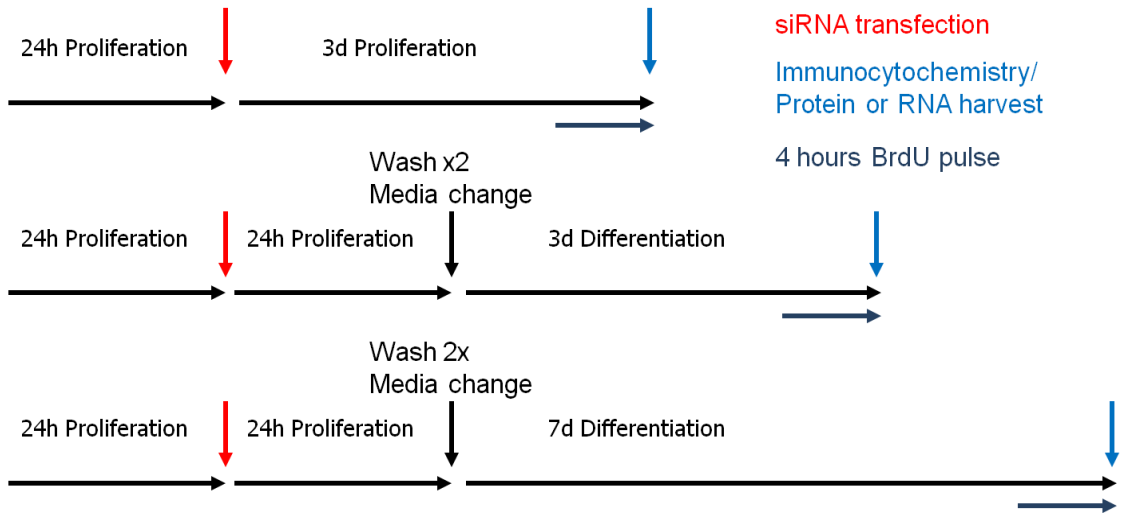


Figure 4-14 Timeline for siRNA transfection of HPCA07/03A cells.
For the proliferation assay (line 1) cells were transfected using the N-TER Nanoparticle siRNA Transfection System 24h after seeding and maintained for 3 days. For the differentiation assay (line 2 and 3) cells were kept for 24h under proliferation was started and maintained for 3 or 7 days.

4.3.1 Klotho mRNA levels are very low before Klotho knock down

To confirm *Klotho* knock down on mRNA level I used several SYBR green primers as well as TaqMan probes. Both failed to deliver accurate results in biological triplicates in the knock down samples as *Klotho* already has a rather low expression in HPC03A/07 cells. The cycle threshold (CT) for *Klotho* after siRNA knock down is very high (~CT 35) meaning it is expressed at a very low level and the results are out of the dynamic range, therefore an accurate analysis of mRNA levels could not be carried out. Nonetheless, Figure 4-15 shows three technical replicates of *Klotho* mRNA expression after *Klotho* knock down. In the siRNA III group mRNA levels have been decreased by a fold change of 0.9. These are not satisfying Q-PCR data, therefore it was decided to validate *Klotho* knock down by assessing Klotho protein expression by immunohistochemistry for each siRNA experiment as shown further in Figure 4-16a,b; Figure 4-17a,b; Figure 4-19a,b.

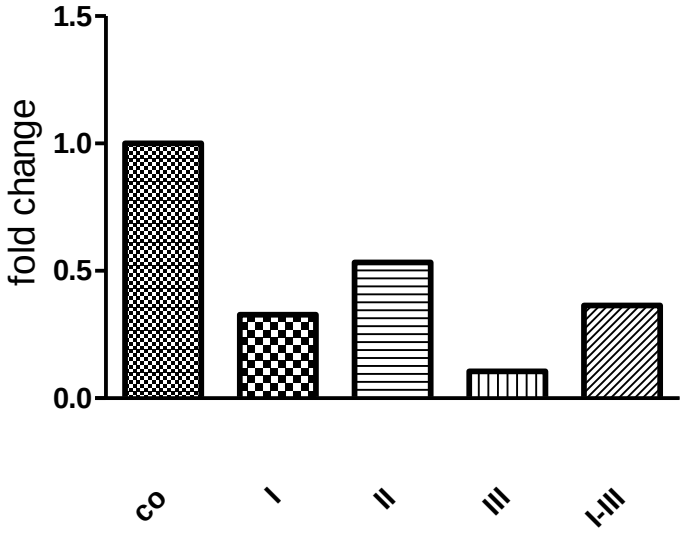


Figure 4-15 *Klotho* Q-PCR in differentiating HPC03A/07 upon *Klotho* knock down
Klotho mRNA levels are reduced to a 0.9 fold change after *Klotho* knock down with siRNA III. Three technical replicates have been used

4.3.2 Attempt to Klotho knock down has no significant effects on proliferating HPC03A/07

Cultures were fixed with 4% PFA and then stained immunocytochemically for Klotho, the proliferation markers BrdU and Ki67 and the NPC markers SOX2 and Nestin. Expression of *Klotho* decreases slightly upon transfection with siRNA but this change is not significant (Figure 4-16a,b). Similarly, the reduction in percentage of Ki67 positive cells was not significant (Figure 4-16c,d). There was no change in SOX2 (Figure 4-16e,f) and Nestin expressing cells (Figure 4-16g,h). Each siRNA separately showed variability in knockdown efficiency and the combination of all three siRNAs is most effective in Ki67, SOX2 and Nestin stained cultures. None of the results are significant which could be because three days is too short for the remaining Klotho protein that was produced in the first 24h to be degraded after the knock down inhibits its further production.

Together these results suggest that during proliferation, *Klotho* RNAi is not sufficiently efficient to significantly decrease the percentage of Klotho protein expressing cells. This might explain only a minor influence of *Klotho* on proliferation as already indicated by the non significant increase in Ki67 positive cells in proliferating and differentiating Klover cells in Section 4.2. The experiment in Klover ON cells show that the effect of *Klotho* on cell development was more pronounced under differentiation conditions. Therefore I next investigated the impact *Klotho* knock down on HPC03A/07 cells after 7 days of differentiation.

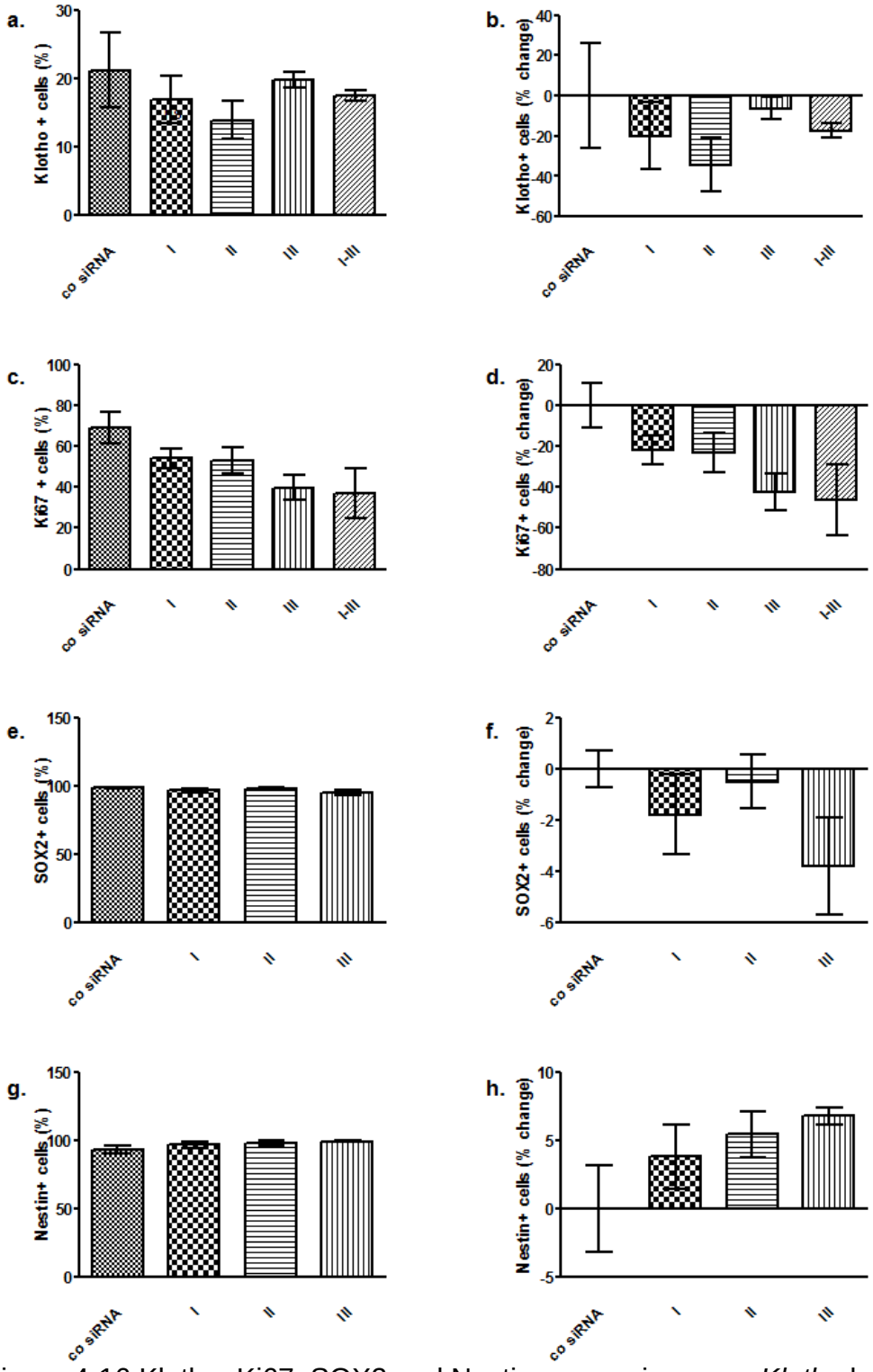


Figure 4-16 Klotho, Ki67, SOX2 and Nestin expression upon *Klotho* knock down in proliferating HPC03A/07 cells

a, c, e, g depict the percentage of positive cells relative to absolute numbers, b, d, f, h show the percentage change of the marker relative to control transfected cells. a) and b) Percentage of Klotho expressing cells c) and d) Percentage of Ki67 expressing cells. e) and f) Percentage of SOX2 expressing cells g) and h) Percentage of Nestin expressing cells. Abbreviationss: Co siRNA: control siRNA not binding to mRNA; I: siRNA binding Klotho mRNA at Exon 2; II: siRNA binding Klotho mRNA at Exon 3; III: siRNA binding Klotho mRNA at Exon 5; I-III: mixture of the three siRNAs. (One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM).

4.3.3 Klotho knock down reduces neuronal commitment in HPC03A/07 after 3 days differentiation

For the 3 days of differentiation experiments, cultures were treated and stained as described in Section 4.3. The proportion of Klotho expressing cells was significantly decreased upon *Klotho* knockdown with siRNAI -54.5±1.8% $p<0.001$, siRNAII -32.66±4.2% $p<0.01$, siRNAIII -21.2±11% $p<0.05$, siRNAI-III -61.2±2.4% $p<0.001$ (Figure 4-17a,b). The percentage of cells expressing Ki67 showed a non-significant decrease upon transfection (Figure 4-17c,d). The proportion of Dcx expressing cells was significantly decreased with siRNAII by -16.9±3.9% $p<0.05$, but not significantly decreased with the other siRNAs (Figure 4-17e,f). The percentage of MAP2 expressing cells was significantly decreased with siRNAI-III by -20.0±3.0% $p<0.05$ but not significantly decreased with the other siRNAs (Figure 4-17g,h). S100b was not significantly decreased (Figure 4-18a,b). Activated Caspase-3 in turn shows not significant increase upon *Klotho* knock down (Figure 4-18c,d). There was no change in the percentage of cells expressing the NPC markers SOX2 and Nestin expressing cells (See Figure 4-18e,f,g,h).

The percentage of expression of the marker for neuronal differentiation Dcx and MAP2 was significantly decreased with siRNAII and siRNAI-III respectively. These data suggest that *Klotho* plays a role in neuronal fate determination in HPC03A/07 cells. To investigate further the role of *Klotho* during differentiation cultures were next maintained under differentiation conditions for a longer period (7 days).

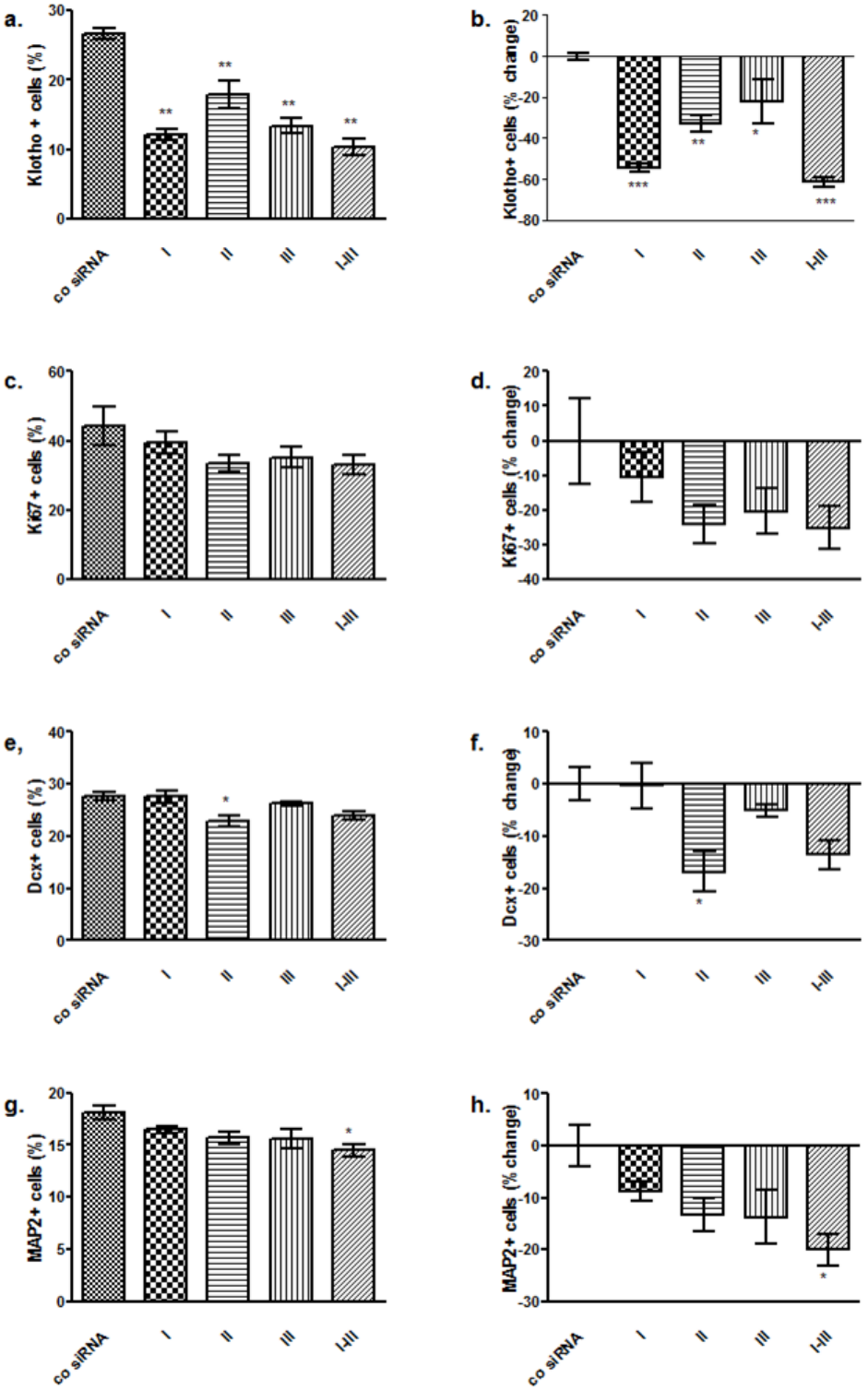


Figure 4-17 Klotho, Ki67, Dcx and MAP2 expression upon *Klotho* knock down after 3 days differentiation in HPC03A/07 cells

a, d, e show the percentage of positive cells relative to absolute cells numbers. b, d, f show the percentage change of the marker in Klotho knock down cells. a) and b) Klotho expressing cells. c) and d) Ki67 expressing cells. e) and f) Dcx expressing cells. g) and h) MAP2 expressing cells. Abbreviations: Co siRNA: control siRNA not binding to mRNA; I: siRNA binding Klotho mRNA at Exon 2; II: siRNA binding Klotho mRNA at Exon 3; III: siRNA binding Klotho mRNA at Exon 5; I-III: mixture of the three siRNAs. (One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM); *p<0.05, **p<0.01)

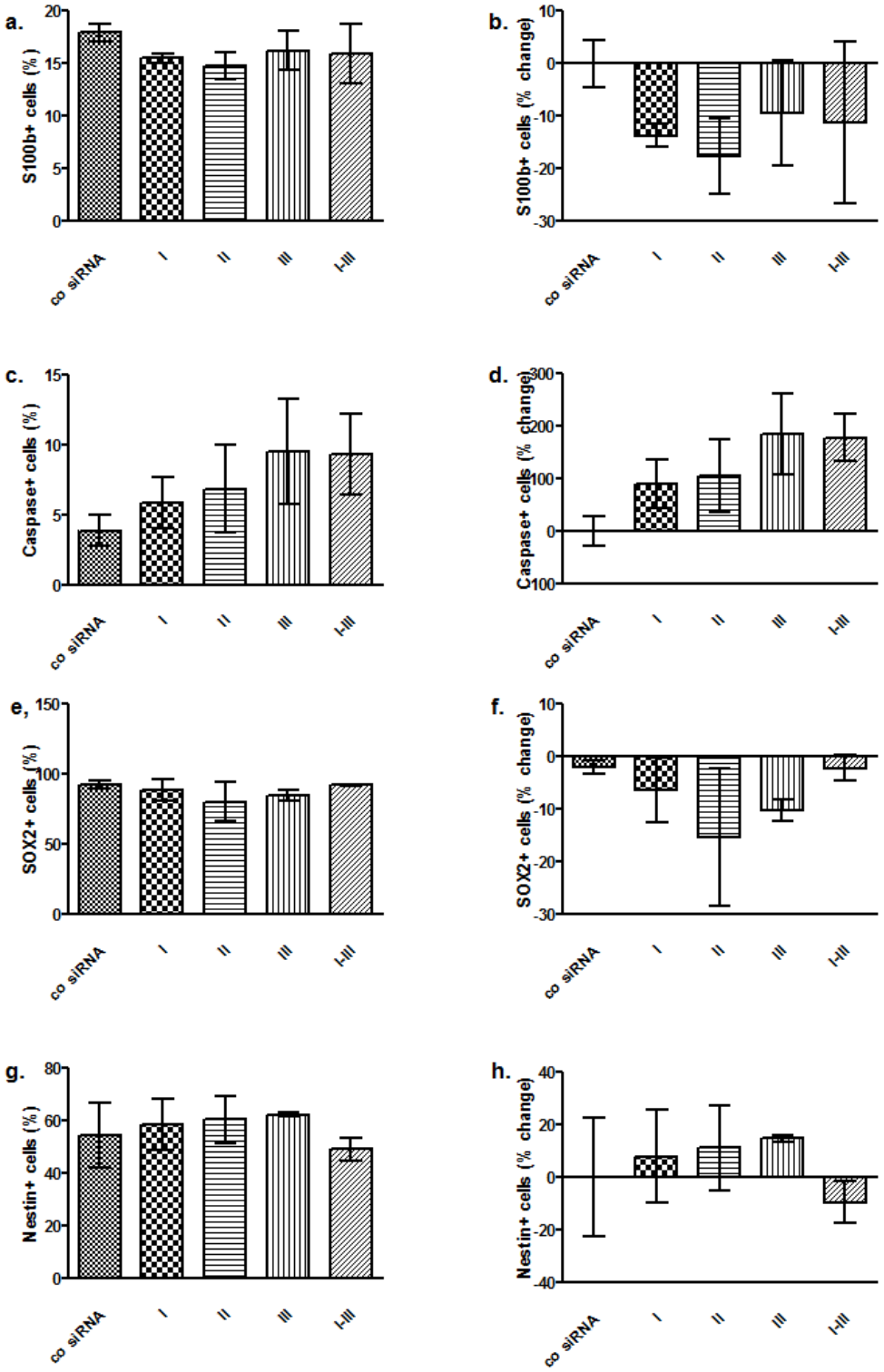


Figure 4-18 S100b, activated Caspase-3, SOX2 and Nestin expression upon *Klotho* knock down after 3 days differentiation in HPC03A/07

a, d, e show the percentage of positive cells relative to absolute cells numbers. b, d, f show the percentage change of the marker in Klotho knock down cells. a) and b) S100b expressing cells. c) and d) activated Caspase-3 expressing cells. e) and f) SOX2 expressing cells. g) and h) Nestin expressing cells. Abbreviations: Co siRNA: control siRNA not binding to mRNA; I: siRNA binding Klotho mRNA at Exon 2; II: siRNA binding Klotho mRNA at Exon 3; III: siRNA binding Klotho mRNA at Exon 5; I-III: mixture of the three siRNAs. (One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM).

4.3.4 Klotho knock down increases apoptosis and decreases neuronal commitment in HPC03A/07 after 7 days differentiation

For the 7-day differentiation experiments, cultures were treated and stained as described in Section 4.3. After 7 days of differentiation Klotho expression was significantly decreased upon *Klotho* knockdown (siRNAI -51.2±0.4%; siRNAII -39.5±6.1%; siRNAIII by -44.7±4.4%; siRNA I-III by -67.4±1.4; all $p<0.001$) (Figure 4-19). Ki67 expression was not significantly decreased upon transfection (Figure 4-20). Dcx expression was significantly decreased with siRNAII -16.5±2.9% $p<0.01$ and siRNAI-III by 18.11±5.8%, $p<0.01$, but not significantly with siRNAI and siRNA III (Figure 4-21a-c) and MAP2 was significantly decreased when transfected with siRNAIII by -15.2±5.9%, $p<0.05$ but not significantly with siRNAI, siRNAII, siRNAI-III (Figure 4-21d-f). S100b, a glial marker, was not significantly decreased (Figure 4-22). Activated Caspase-3 in turn showed a significant increase upon *Klotho* knock down (siRNAI by 95.6±12.8%, $p<0.05$; siRNAII by 117.1±12.0%, $p<0.05$; siRNAIII by 108.0±21.3%, $p<0.05$; siRNAI-III by 163.6±34.9%, $p<0.01$) (Figure 4-23). The proportion of SOX2 and Nestin expressing cells is not significantly changed.

The markers for neuronal differentiation Dcx and MAP2 significantly decreased with siRNAII and siRNAIII respectively and the proportion of apoptotic cells was significantly increased. This suggests that Klotho is necessary for the survival of differentiating cells. It appears that the neuronal cells develop but then die as the percentage of neuronal cells is the only population that is significantly decreased (apart from the Klotho population). However to confirm what cell types die it will be necessary to co-label cultures for activated Caspase-3 and marker for proliferation, differentiation and astrocytosis. Together with the data from 3 days proliferation (Section 4.3.1) and 3 days differentiation after knock

down (Section 4.3.4) these data indicate that Klotho is important during early (3 days) and more advanced (7 days) differentiation and survival in HPC03A/07 cells. However, it seems as if Klotho has no effect on dividing cells. As shown earlier (4.3.1) *Klotho* expression is very low and therefore a knockdown of *Klotho* might not have as big as an impact as over expressing it.

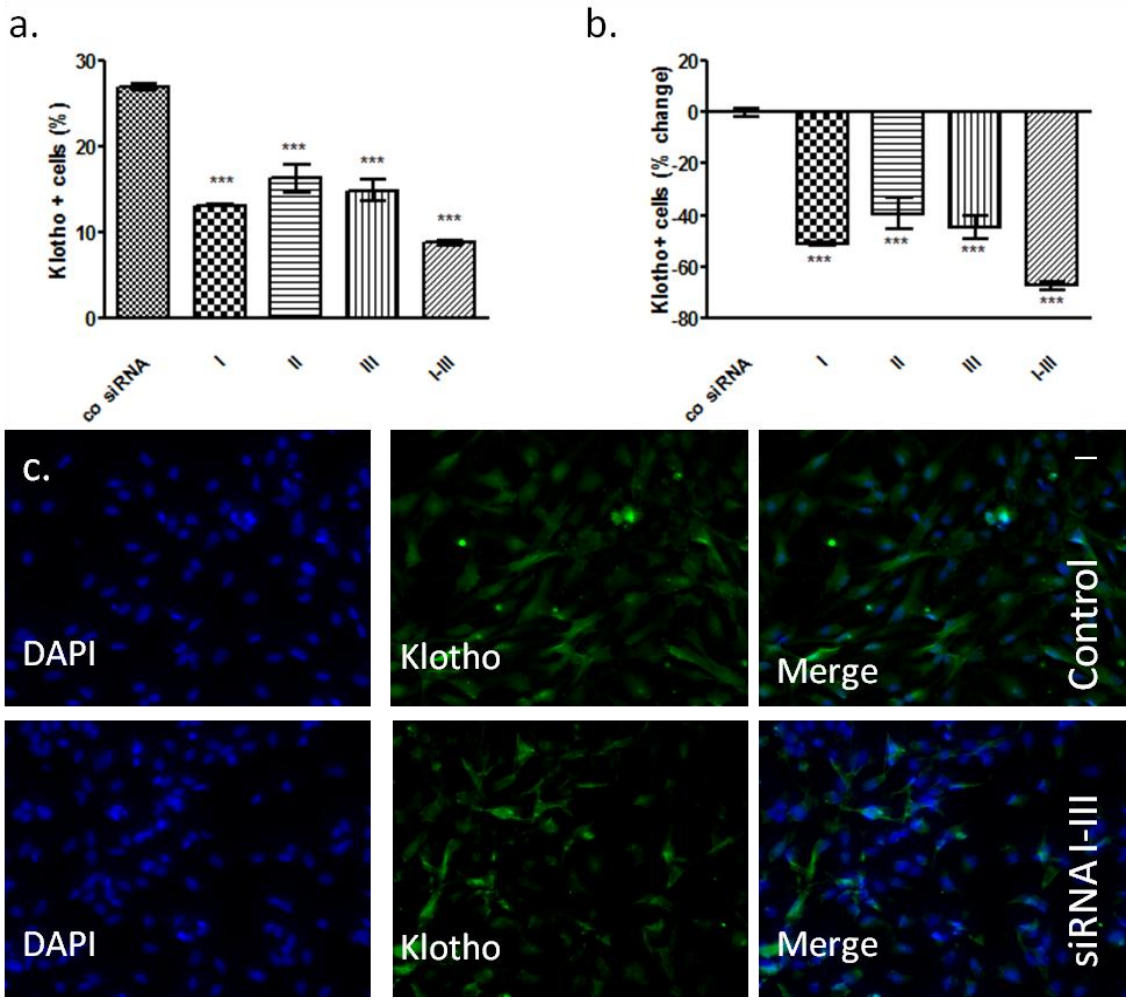


Figure 4-19 Klotho expression in differentiating HPC03A/07 upon Klotho knock down

a) Comparison of the absolute percentage of Klotho positive cells in knock knock down and control cells. b) Percentage change of Klotho expression compared to the control c) Images show in the top row cultures transfected with control siRNA, bottom row shows cultures transfected with a mix of the three siRNAs. Abbreviations: siRNA: control siRNA not binding to mRNA; I: siRNA binding Klotho mRNA at Exon 2; II: siRNA binding Klotho mRNA at Exon 3; III: siRNA binding Klotho mRNA at Exon 5; I-III: mixture of the three siRNAs. (Scalebar: 20µm, One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM); ***p<0.001)

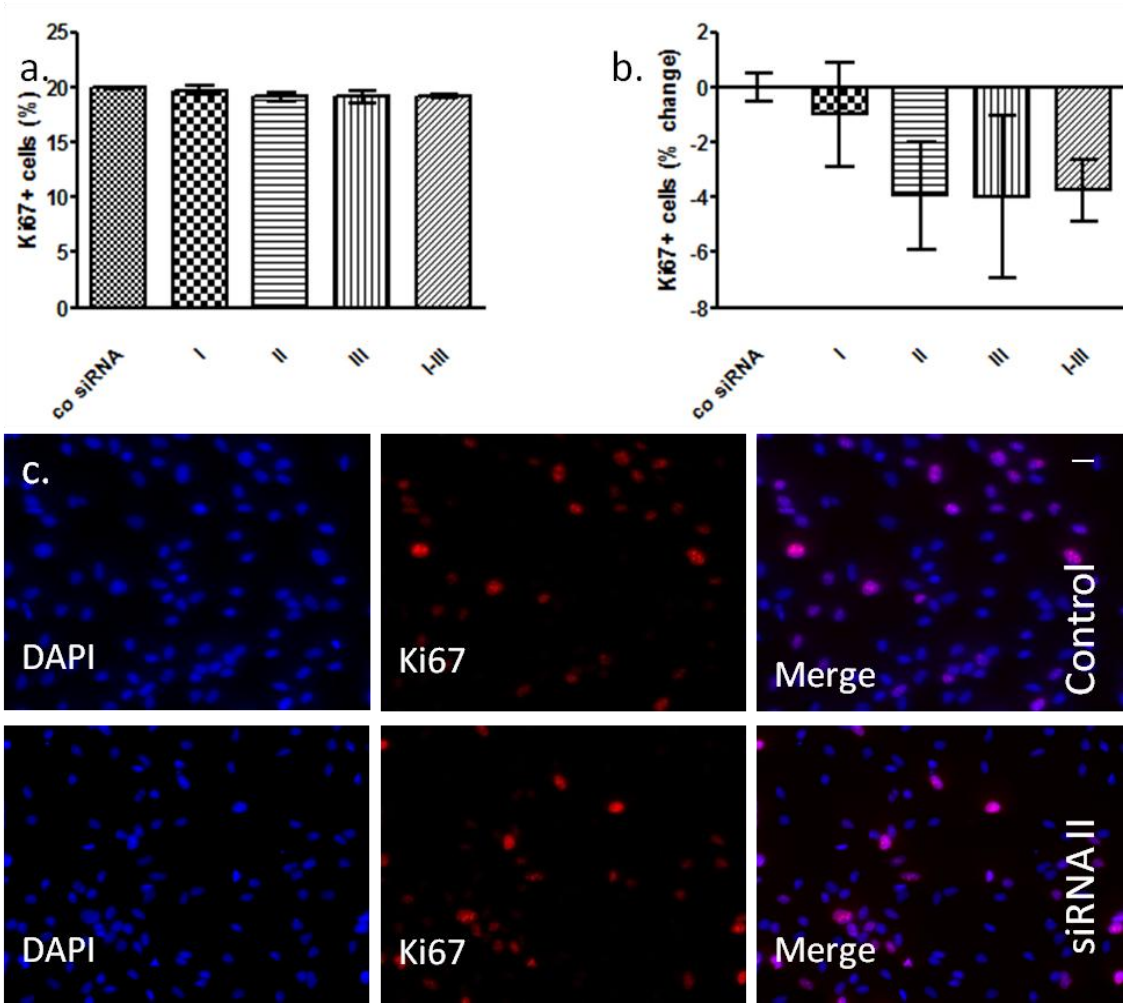


Figure 4-20 Ki67 expression in HPC03A/07 upon Klotho knock down

a) Comparison of the absolute percentage of Ki67 positive cells in knock knock down and control cells. b) Percentage change of Ki67 expression compared to the controlc) Images show in the top row cultures transfected with control siRNA, bottom row shows cultures transfected with a mix of the three siRNAs. Avreviations: Co siRNA: control siRNA not binding to mRNA; I: siRNA binding Klotho mRNA at Exon 2; II: siRNA binding Klotho mRNA at Exon 3; III: siRNA binding Klotho mRNA at Exon 5; I-III: mixture of the three siRNAs. (Scalebar: 20µm, One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM))

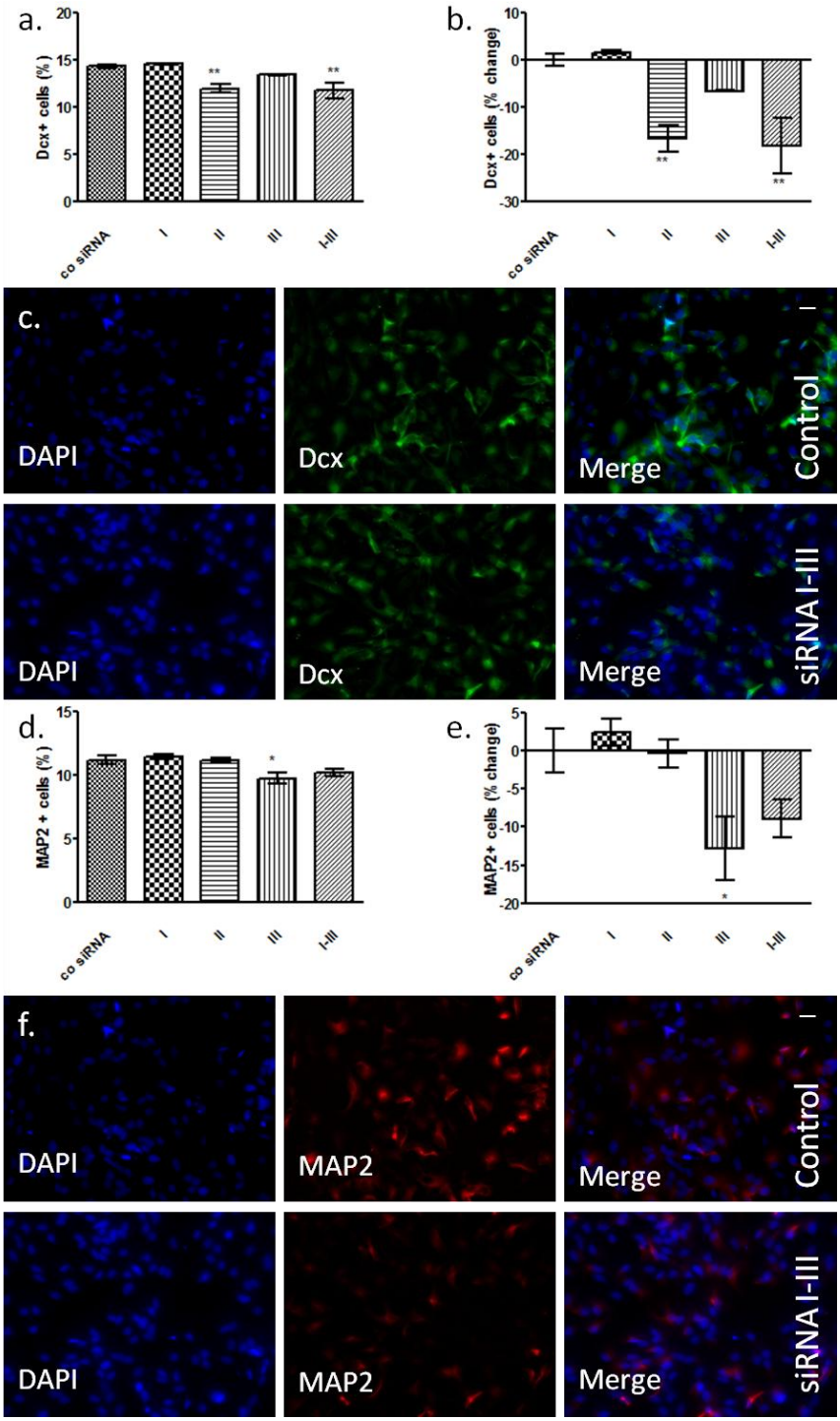


Figure 4-21 Dcx and MAP2 expression in differentiating HPC03A/07 cells
a, e show the percentage of positive cells relative to absolute cells numbers. d, e show the percentage change of the marker in Klotho knock down cells a) Comparison of the absolute percentage of Dcx positive cells in knock down and control cultures. b) Percentage change of Dcx expression compared to control d) Comparison of the absolute percentage of MAP2 positive cells in knock down and control cultures. e) Percentage change of MAP2 expression compared to the control c) and f) Images in the top row show control transfected cultures, bottom row shows Klotho knock down. Abbreviations: co siRNA: control siRNA not binding to mRNA; I: siRNA binding Klotho mRNA at Exon 2; II: siRNA binding Klotho mRNA at Exon 3; III: siRNA binding Klotho mRNA at Exon 5; I-III: mixture of the three siRNAs. (Scalebar: 20µm; Student's t-test, Error bars are the Standard Error of the Mean (SEM); *p<0.05, **p<0.01, ***p<0.001)

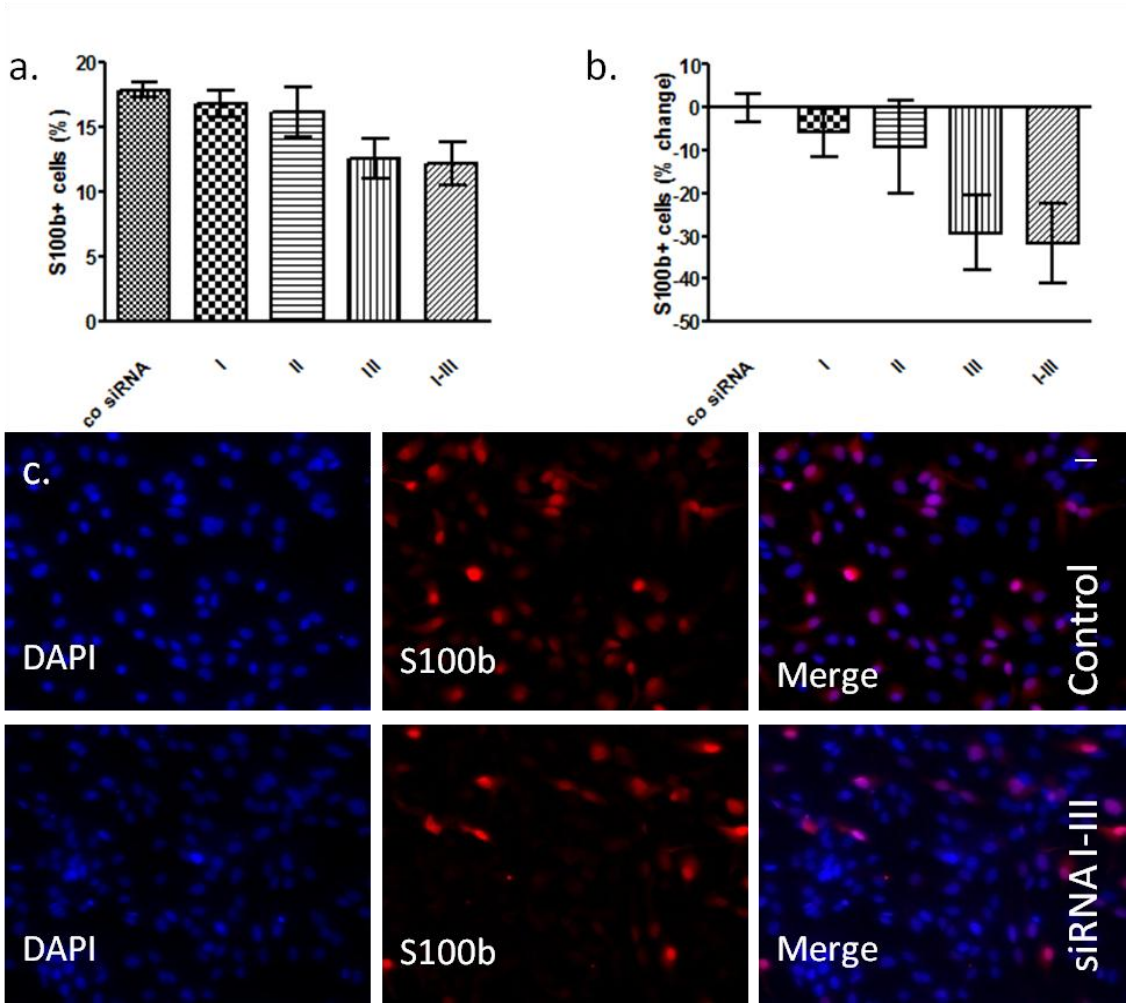


Figure 4-22 S100b expression in differentiating HPC03A/07 upon Klotho knock down

a) Comparison of the absolute percentage of S100b positive cells in knock down and control cells. b) Percentage change of S100b expression compared to the control c.) Images show in the top row cultures transfected with control siRNA, bottom row shows cultures transfected with a mix of the three siRNAs. Abbreviationss: Co siRNA: control siRNA not binding to mRNA; I: siRNA binding Klotho mRNA at Exon 2; II: siRNA binding Klotho mRNA at Exon 3; III: siRNA binding Klotho mRNA at Exon 5; I-III: mixture of the three siRNAs. (Scalebar: 20µm, One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM))

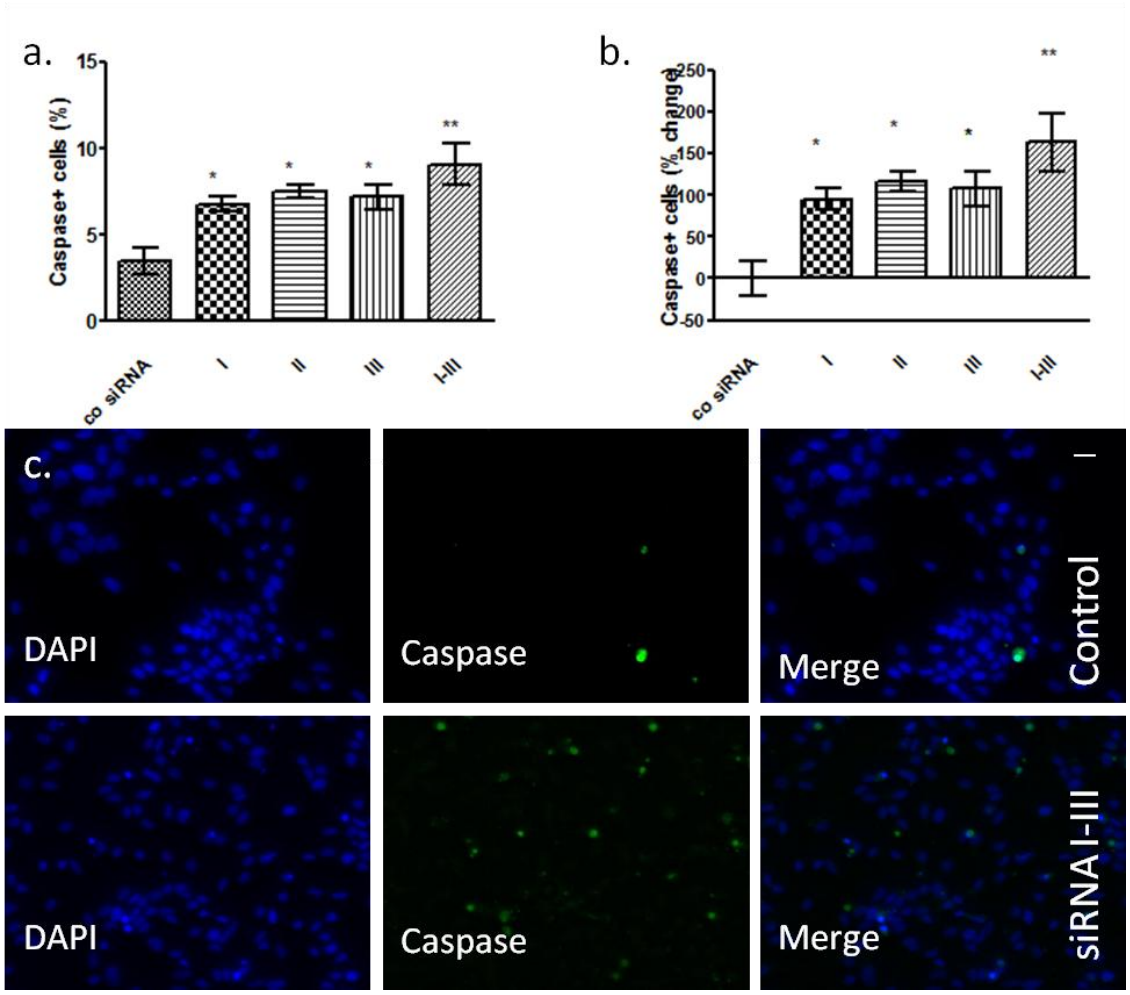


Figure 4-23 Activated Caspase-3 expression in differentiating HPC03A/07 upon Klotho knock down

a) Comparison of the absolute percentage of activated Caspase-3 positive cells in knock knock down and control cells. b) Percentage change of activated Caspase-3 expression compared to the control c) Images show in the top row cultures transfected with control siRNA, bottom row shows cultures transfected with a mix of the three siRNAs. Abbreviations: Co siRNA: control siRNA not binding to mRNA; I: siRNA binding Klotho mRNA at Exon 2; II: siRNA binding Klotho mRNA at Exon 3; III: siRNA binding Klotho mRNA at Exon 5; I-III: mixture of the three siRNAs. (Scalebar: 20µm, One-Way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM); *p<0.05, **p<0.01,)

4.3.5 Summary

Over-expression of *Klotho* in proliferating Klover cells increases translocation of SOX2 in the cytoplasm but has no notable effect on apoptosis or proliferation. During differentiation *Klotho* over-expression increases apoptosis as well as the proportion of neuronal cells and astrocytic cells. These data together with the increase in SOX2 cytoplasm positive cells suggest that *Klotho* over-expression pushes the cultures into cell fate commitment. It is possible that over expressing the secreted form of *Klotho* accelerates fate commitment to the point of inducing more rapid cell death. It would be interesting to investigate which cell types are dying by co-labelling cells for activated Caspase-3 together with neuronal, astrocytic or NPC marker. *Klotho* over-expression appears to affect neuronal and astrocytic development as well as cell survival but has no effect on the proportion of dividing cells.

A *Klotho* knock down on the other hand was expected to have the opposite effect. However, mRNA levels of *Klotho* are already very low before *Klotho* knock down. It was therefore difficult to further decrease *Klotho* expression and reliably quantify the extent of the knock down especially for the proliferation experiments. Indeed, during proliferation, *Klotho* knock down does not lead to any significant decrease in the percentage of Klotho, Ki67, SOX2 or Nestin positive cells, suggesting that the diminishing *Klotho* does not have a major influence on HPC03A/07 cells during proliferation. The not significant decrease in the percentage of Klotho expressing cells might be due to the fact that after 3 days of proliferation after the knock down, previously generated Klotho protein has not been degraded yet. However, after 3 days of differentiation the proportion of Klotho expressing cells was significantly decreased suggesting that Klotho turnover is higher in differentiating cells and that its expression

affects cell development during differentiation rather than proliferation. Moreover, after 3 days of differentiation the proportion of neuronal cells was decreased when transfected with siRNAII or I-III, further suggesting a role for *Klotho* during neuronal development. This is confirmed during more advanced (7 days) differentiation where *Klotho* knock down leads to a significant decrease in Klotho and early and mature neurons, whereas apoptosis was significantly increased, suggesting that Klotho is required for cell survival upon differentation. To quantify what cell type dies, double labelling with activated Caspase-3 and marker for proliferation, neurons and astrocytes will be necessary.

Together, *Klotho* over-expression and *Klotho* knock down indicate that *Klotho* is required for neuronal development and survival but leaves proliferation unaffected.

4.4 Discussion

4.4.1 Klotho is expressed in mouse hippocampus in neurons and stem cells

My results in 4.1 show that in wild type mouse brain, Klotho protein is expressed in some neurons ($NEUN^+$) and stem cells ($SOX2^+$), but not in glial cells ($GFAP^+$), NPC ($Nestin^+$), dividing cells ($Ki67^+$) and neuroblasts (Dcx^+), suggesting that Klotho is only expressed in mature neurons and SOX2 positive stem cells. To date the majority of research regarding *Klotho* has been conducted in kidney, liver or the cardiovascular system (Kuro-o, 2011; Maltese, 2012) and sofar my study is the first to specify which cell types in the brain express Klotho.

The present study in this thesis shows for the first time that Klotho protein is expressed in the DG of the hippocampus, which correlates with the presence of *Klotho* mRNA in the same region. Klotho protein is not expressed in GFAP positive cells in the mouse hippocampus nor in S100b positive cells in the human hippocampal cell line. Interestingly, Klotho was detected in ependymal cells off the SVZ (German et al., 2012). Ependymal cells are a type of glial cells typically expressing GFAP and/or S100b, illustrating that the Klotho population in the DG is likely made of different cell types than the Klotho positive population near the SVZ. Further, my data show that in the DG, Klotho is expressed in SOX2 but not in GFAP positive cells. Interestingly, self-renewing nonradial progenitors in the SGZ, the type 2 cells, are $SOX2^+$ but $GFAP^-$ and give rise to Dcx^+ neuroblasts that differentiate into dentate granule cells (Mu et al., 2010). This might be an indication that Klotho is expressed in type 2 cells in the DG, suggesting a putative role of Klotho for cell differentiation.

4.4.2 Intermittent fasting increased the amount of Klotho protein in the dentate gyrus but not the number of Klotho positive cells.

My results in 4.1.1 show that intermittent fasting (IF) increases the amount of protein that the existing Klotho positive cells produce in the dentate gyrus of mice but that the total proportion of Klotho expressing cells did not increase. A difference in Klotho intensity was also shown by German et al in the choroid plexus of wild type, *Klotho* mutant and *Klotho* over-expressing mice. Klotho expression in the organs of *Klotho* over-expressing mice is very intense compared to wild type and *Klotho* mutant mice (German et al., 2012). The difference in the intensity of Klotho expression was also seen in unpublished data by the Thuret lab described in the introduction (1.3.4) demonstrating that *Klotho* mRNA was increased twofold in the hippocampus of mice maintained on an IF diet. These animals displayed increased neurogenesis and proliferation in the DG and improved associated behaviour. IF, *Klotho* and RSVL show similar effects on lifespan and age related diseases, namely increasing lifespan and ameliorating the symptoms of age related diseases (Kurosu et al., 2005; Pearson et al., 2008; Dolinsky and Dyck, 2011; Mercken et al., 2011). The increase in mRNA and Klotho protein expression in the brain without increasing the number of Klotho positive cells underpins the endocrine, humoral activity of the secreted form of Klotho explaining the general phenotype in *Klotho* mutant mice despite the organ-restricted expression (Kuro-o et al., 1997; Nagai et al., 2003). Using heterochronic parabiosis Villeda et al. show that blood-borne factors present in the systemic milieu can inhibit or promote adult neurogenesis in an age-dependent fashion in mice. Accordingly, exposing a young mouse to an old systemic environment or to plasma from old mice decreased synaptic plasticity, and impaired contextual fear conditioning and spatial learning and

memory. This confirms that the decline in neurogenesis and cognitive impairments observed during ageing can be in part attributed to changes in blood-borne factors (Villeda et al., 2011).

4.4.3 Klotho over expression increases neurogenesis but has no effect on dividing cells

My data show in (4.2) that over-expression of *Klotho* in HPC03A/07 increases neurogenesis and apoptosis during differentiation but has no effect on proliferating cells. *Klotho* appears to be involved in neurogenesis and survival; however it seems to have no effect on dividing cells and proliferation. It appears *Klotho* did not show any effects on proliferation in HEK-293 cells (Chen et al., 2010), which is in accordance with my data that neither *Klotho* over-expression nor *Klotho* knock down affect dividing cells.

Klotho has marked effects on insulin physiology by inhibiting insulin/IGF1R signalling (Kurosu et al., 2005). Negative regulation of insulin/IGF1R signalling is an evolutionarily conserved mechanism to suppress aging and extends life span in yeast, nematodes, fruit fly and rodents (Barbieri et al., 2003; Tatar et al., 2003). Endogenous IGF1 plays an important role for neurogenesis in the adult rat brain development (Anderson et al., 2002; Koltai et al., 2011) and during brain development in human (Netchine et al., 2011). *Klotho* inhibits IGF1 signalling and thereby it appears to be increasing lifespan but decreasing neurogenesis and thereby ensuring the availability of stem cells in the brain into ripe old age, similar to Wnt which is discussed below. This mechanism could explain why *Klotho* over-expression in my *in vitro model* has no effect on the percentage of dividing cells. However, data from the Vischer lab suggested that decreased *Klotho* expression is not a general feature of rodent models of insulin

resistance and that the soluble Klotho protein does not inhibit IGF-1 and/or insulin signalling in HEK293, L6 and HepG2 cultured cells, arguing against a direct role of *Klotho* in insulin signalling (Lorenzi et al., 2010).

Additionally apoptosis is increased in the Klover ON cells in this thesis. Strong mitogen stimulation such as *Klotho* over-expression can cause growth arrest and senescence by over-activating mitogen-activated pathways. In primary cells, the Ras/Raf/MEK/MAPK pathway is initially mitogenic but eventually induces premature senescence. (Lin et al., 1998). This over-stimulation could be a potential explanation for the increase in cell death in the *Klotho* over-expressing cultures in my results.

4.4.4 Klotho is required for differentiation and survival but not dividing cells

My results in 4.3 show that *Klotho* is required for neuronal differentiation and survival during differentiation but is not important during proliferation and for dividing cells. It is plausible that *Klotho* is involved in the regulation of brain aging: *Klotho* mutant mice have a reduced number of Purkinje cells in the cerebellum but no other age-related changes such as senile plaques or amyloid deposits (Nagai et al., 2003). Further, in *Klotho* mutant brain, reduction of synapses was evident in the hippocampus (Li et al., 2004). However, a role during development seems unlikely, because *Klotho* mutant mice develop normally up to at least 2 weeks of age in both macroscopic and histological appearance, therefore the phenotypes seen in *Klotho* mutant mice cannot simply be a result of incomplete development (Kuro-o et al., 1997). This supports the diminishing effect on neuronal differentiation in the *in vitro* knock down model in this thesis in 4.3.3. To further corroborate the importance of

Klotho during differentiation *Klotho* promoted differentiation in adipocytes during the period of transient proliferation by elevating mRNA levels of adipocyte differentiation marker, such as PPARγ (Chihara et al., 2006). In 5.6.1 I will further discuss the link between *Klotho* and PPARγ in HPC03A/07 cells.

My results also show an increase in apoptosis following *Klotho* knock down which is in line with the findings in *Klotho* mutant mice (Nagai et al., 2003). Interestingly, the increase in apoptosis in the hippocampus of *Klotho* mutant mice is caused by an increase in lipid peroxide and oxidative damage to DNA (Nagai et al., 2003). In turn *Klotho* in *Klotho* over-expressing mice increases the resistance to oxidative stress involving the ASK1-signalosome via the p38 MAPK pathway (Hsieh et al., 2010). It would be worth investigating what cell type dies in the HPC03A/07 cell line.

The changes in cellular composition following reduced expression of *Klotho* in *Klotho* mutant or old mice lead to behavioural changes in rodents that mirror the phenotype of human aging. *Klotho* mutant mice display impaired long-term, but not short-term retention of novel object recognition and fear conditioning at 7 weeks but not at 6 weeks (Nagai et al., 2003). The findings from the Thuret lab described in the introduction 1.3.4 show a similar effect although the other way around: IF mice with increased *Klotho* level in the hippocampus display improved long-term but not short-term retention memory. These data are in accordance with the decreased neurogenesis described in depressed patients (Stanley Medical Research Institute ID6322) and my data demonstrating that a decrease of *Klotho* leads to decreased neurogenesis. Further, a polymorphism in the *Klotho* gene in young and aged human is associated with lower cognitive ability (Deary et al., 2005).

To understand better how *Klotho* elicits its effect on neurogenesis and survival in HPC03A/07 it would be necessary to investigate which cell types are dying by co-staining cells for activated Caspase-3 with marker for proliferation (BrdU), neuroblasts (Dcx) and neurons (MAP2). Further, to elucidate the cellular mechanism behind the effects of *Klotho* on differentiation, it would be interesting to investigate changes in Wnt expression upon *Klotho* over-expression and knock down. Wnt can be bound by the secreted form of Klotho (Kirstetter et al., 2006; Scheller et al., 2006; Liu et al., 2007), which leads to suppression of Wnt activity when *Klotho* is over-expressed and an increase in Wnt signalling in *Klotho* mutant mice. Continuous Wnt exposure triggered accelerated cellular senescence (Liu et al., 2007). Wnt over-expression triggers neurogenesis in the adult brain (Lie et al., 2005). The suppression of Wnt by *Klotho* might protect the stem cell pool from depletion and ensure the availability of self-renewing cells to a ripe age.

Further, the Insulin/IGF1 pathway would be worth a closer examination to elucidate the conundrum between its effect on aging and neurogenesis as described above. This should preferably be investigated in an animal model to be able to assess the effects of aging properly. Obviously it would be very interesting to thoroughly examine proliferation and differentiation in *Klotho* mutant and *Klotho* over-expressing mice and further behavioural tests to investigate the role of *Klotho* in learning/memory and depression as so far only one group looked at and confirmed cognitive impairment in *Klotho* mutant mice (Nagai et al., 2003).

Chapter 5 The proliferative and neurogenic effects of Resveratrol on neural stem cells are partly dependant on Klotho and PPARγ

In Section 3.2 I have shown that RSVL increases the number of dividing and neuronal cells. I now wish to determine whether this effect is mediated by *Klotho* via PPARγ.

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes (Greene et al., 1995). PPARs play essential roles during cellular differentiation, development, cell metabolism and tumorigenesis (Fajas et al., 1997). Activation of PPARγ regulates, amongst a variety of other genes and pathways, neural stem cell proliferation and differentiation *in vivo* and *in vitro* (Morales-Garcia et al., 2011). PPARγ activates transcription of *Klotho* in HEK293 cells (Zhang et al., 2008). Moreover, PPARγ can be activated by RSVL in murine primary cortical cultures, bovine brain microvessel vascular endothelial cells (BBMEC), human umbilical venous endothelial cells (HUVEC) and bovine arterial endothelial cells (BAEC) (Inoue et al., 2003). PPARγ and *Klotho* therefore potentially provide another link between diet (RSVL) and AHN, where I hypothesise that RSVL exerts its neurogenic effect via PPARγ and *Klotho*. The experiments within this chapter aim to test this hypothesis by determining whether RSVL (1) increases the percentage of Klotho expressing cells, (2) acts via PPARγ to increase the percentage of HPC03A/07 cells that express Klotho and (3) acts via PPARγ and Klotho to influence the division and neurogenesis of HPC03A/07 cells.

5.1 RSVL increases the number of Klotho expressing cells in proliferating and differentiating HPC03A/07

To assess the effect of RSVL on the proportion of Klotho expressing cells, cells were cultured as described in Section 2.1.1. 24h after seeding, cultures were treated with 1µM RSVL and maintained for under proliferative conditions for the proliferation assay. For the differentiation assay differentiation was started 24h after seeding and RSVL was added at the same time. Cultures were maintained for 7 days under differentiation conditions. At the end of each experiment, cultures were fixed with 4% PFA for immunocytochemical staining. Cells were stained for Klotho protein, the proliferative activity marker Ki67, the neuroblasts marker Dcx and the mature neurons marker MAP2.

Treatment with 1µM RSVL for 3 days under proliferation conditions significantly increased the percentage of Klotho positive cells in the cultures by 119.9±2.7%, $p<0.001$. Also after 7 days of differentiation RSVL significantly increases the percentage of Klotho positive cells by 47.9±2.0%, $p<0.001$ (See Figure 5-3a). This set of data confirms my first hypothesis that RSVL increases the proportion of Klotho expressing cells in proliferating and differentiating HPC03A/07. See Figure 5-3e.

5.2 RSVL partly requires PPARγ to act on Klotho positive cells and neurogenesis but not to act on dividing HPC03A/07 cells

To assess whether RSVL exerts its effect on AHN exclusively via PPARγ, HPC03A/07 were cultured as described in Section 2.1.1, and 24 hours later treated as shown in Figure 2-10 with GW9662, an irreversible PPARγ antagonist. After a further 24h, cultures were treated with RSVL. Cells were then maintained either for 3 days under proliferative conditions or 7 days under differentiation conditions before being fixed with 4% PFA and immunocytochemically stained for Klotho protein, the proliferation marker Ki67 as well as Dcx, a marker for neuroblasts, and MAP2, a neuronal marker.

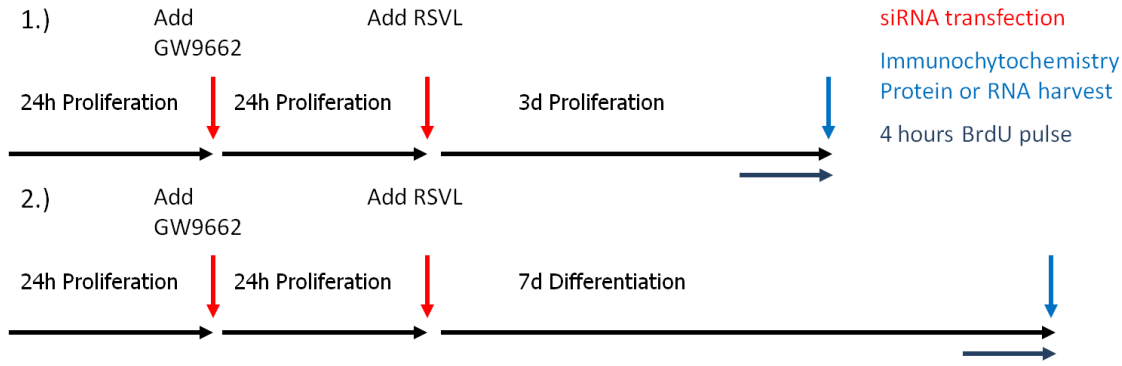


Figure 5-1 Timeline for PPARγ experiment on HPC0A07/03A cells
1.) Proliferation assay, 2) Differentiation assay. Cells were cultured for 24h after seeding before GW9662 was added. After 24h proliferation RSVL was added. For the proliferation assay cells were then maintained under proliferation conditions for 3 days. For the differentiation assay, differentiation was started simultaneously with RSVL treatment and maintained for 7 days.

5.2.1.1 RSVL partly requires PPARγ to activate Klotho

PPARγ was observed to be necessary during proliferative conditions for the activation of *Klotho* expression by RSVL (Figure 5-3b). The percentage of Klotho expressing cells was significantly decreased by -42.4±0.7%, ($p<0.001$) to a total of 18.3±0.2% compared to RSVL (total of 31.7±0.4%) only treated cultures when PPARγ was blocked by GW9662 prior to RSVL treatment. However it was significantly increased compared to the DMSO control with a total of 14.4±0.1%. Also during differentiation PPARγ is required for the activation of *Klotho* expression by RSVL as shown Figure 5-3f. The percentage of Klotho positive cells is significantly decreased when PPARγ is blocked with GW9662 by -21.9±0.2%, $p<0.001$ to a total of 28.8±0.1% compared to RSVL (36.9±0.5%) only treated cultures. However the percentage of Klotho expressing cells was significantly increased compared to the DMSO control (24.9±0.1%)

5.2.1.2 RSVL does not require PPARγ to increase the number of Ki67 expressing cells in proliferating and differentiating HPC03A/07

Here I confirm what I have already demonstrated in 3.2: RSVL treatment significantly increases (31±0.7%, $p<0.01$) the percentage of Ki67 positive cells compared to DMSO treated control cultures during proliferation (Figure 5-4a). Also under differentiation conditions RSVL treatment significantly increases the percentage of Ki67 positive cells by 100.9±2.9%, $p<0.001$, see Figure 5-4a. This confirms that RSVL is able to increase division even under differentiation conditions as demonstrated before.

Figure 5-4b shows that during proliferation PPARγ is not necessary for the activation of Ki67 expression by RSVL. The percentage of Ki67 positive cells

shows no significant change compared to the RSVL treated cultures when PPARγ is blocked with GW9662. Also during differentiation PPARγ is not required by RSVL to increase the percentage of Ki67 positive cells (Figure 5-4f). The percentage of Ki67 positive cells shows no significant changes in either proliferation or differentiation, suggesting that PPARγ is not involved it the activation of dividing cells by RSVL.

5.2.1.3 RSVL partly requires PPARγ to increase neurogenesis in differentiating HPC03A/07

First I confirmed the increase in the percentage of Dcx and MAP2 expressing cells in cultures treated with RSVL. In these experiments, cultures express significantly more Dcx positive cells when compared to control cultures (percentage change of 86.8±3.8%, $p<0.001$, Figure 5-5a) . The changes in the percentage of MAP2 expression was similar to Dcx. Cultures treated with RSVL are composed of significantly more MAP2 positive cells by 91.02±1.2%, $p<0.001$ (Figure 5-5e) compared to the DMSO control. The increase in neurogenesis induced by RSVL that is observed is genuinely due to an increased production of neuronal differentiation and not due to increased survival as RSVL also increases the proportion of apoptotic cells under the same conditions, see Figure 5-6a.

PPARγ is required for RSVL to increase the percentage of Dcx (Figure 5-5b) or MAP2 (Figure 5-5f) expressing cells. Indeed, the proportion of neuroblasts is significantly decreased after RSVL treatment when PPARγ is blocked with GW9662 by -26.6±1.1%, $p<0.001$ to a total of 17.5±0.3% compared to RSVL only treated cultures. This however was significantly increased compared to the DMSO control with 12.7±0.6% ($p<0.001$). Also the proportion of mature neurons

is significantly decreased when PPARγ is blocked with GW9662 by -38.3±2.0%, $p<0.001$ to a total of 23.7±0.8. Compared to the DMSO control (total of 20.1±0.6%) this value was significantly ($p<0.001$) increased.

5.2.1.4 Absence of PPARγ prevents RSVL induced apoptosis in the differentiating HPC03A/07

I first confirmed in this independent set of experiments what I discovered in 3.2: RSVL treatment significantly increases the percentage of activated Caspase-3 expressing cells by 24.2±0.9%. When PPARγ is blocked before RSVL treatment apoptosis is significantly decreased by -15.6±0.3%, $p<0.001$ to a total of 17.5±0.1% compared to RSVL only treated cultures (total of 16.7±0.4%) (Figure 5-6b). This suggests that PPARγ is involved in the apoptotic effect RSVL exerts on HPC03A/07 cells.

5.3 Summary

PPARγ is partly required by RSVL to exert its increasing effect on *Klotho* expression and neurogenesis. PPARγ is not necessary for RSVL induced effects on dividing cells. RSVL induced apoptosis is diminished by the lack of PPARγ.

These results are similar to my results in 4.3, where a *Klotho* knock down affects neurogenesis and apoptosis but not dividing cells in HPC03A/07 cells. Further in my results PPARγ was observed to be necessary for the activation of *Klotho* expression by RSVL during proliferation and differentiation (Figure 5-3b,f).

I therefore went on to investigate the role of *Klotho* downstream of RSVL induced changes to dividing and neuronal cells and apoptosis.

5.4 RSVL partly requires Klotho to increase the number of Ki67 positive cells and neurogenesis

To assess whether *Klotho* was a necessary downstream target of PPARγ for proliferation and neurogenesis, *Klotho* expression was knocked down by transfection with a mixture of three different *Klotho* targeting siRNAs using the N-TER transfection system as explained in 2.1.7. 24h after the siRNA transfection, cultures were treated with either the PPARγ agonist Rosiglitazone or RSVL. Rosiglitazone was used to compare the effects of RSVL to specific PPARγ activation. If RSVL and Rosiglitazone show the same immunocytochemical profile it suggests that RSVL acts exclusively via PPARγ, conversely if significant differences are observed this suggests that RSVL acts independently or only partly via PPARγ. If *Klotho* is uniquely necessary for the action of PPARγ (activated by RSVL or Rosiglitazone) then I would not expect to see an effect in the *Klotho* knock down group. As described above, after completion of the treatments cultures were maintained for either 3 days under proliferative conditions or 7 days under differentiation conditions before being fixed with 4% PFA followed by immunochytochemical staining for the markers stated above (see Figure 5-2 for a cartoon of these experimental procedures).

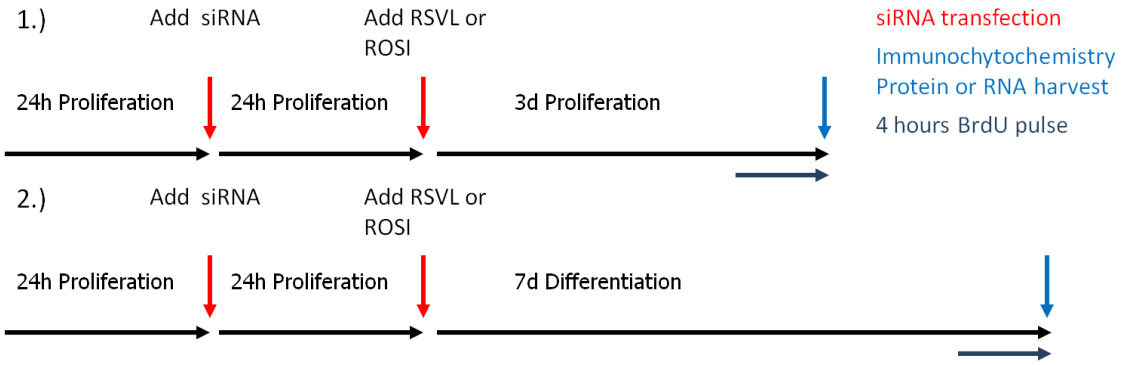


Figure 5-2 Timeline for *Klotho* and PPARγ experiment on HPC0A07/03A cells
1) Proliferation assay, 2) Differentiation assay. Cells were cultured for 24h after seeding before *Klotho* knock-down using siRNA. After 24h proliferation either RSVL or Rosiglitazone (ROSI) were added. For the proliferation assay cells were then maintained under proliferation conditions for 3 days. For the differentiation assay, differentiation was started simultaneously with RSVL or ROSI treatment and maintained for 7 days.

For the *Klotho* knock down *Klotho* expression was knocked down by transfection with a mixture of three different *Klotho* targeting siRNAs using the N-TER transfection system as explained in Section 2.1.7. 24h after seeding. For the proliferation assay cells were maintained for 3 days under proliferation conditions, for the differentiation assay differentiation was started 24h after the transfection and cultures were maintained for 7 days under differentiation conditions. To demonstrate a valid *Klotho* knock down for this set of experiments, cells were also stained for Klotho protein upon siRNA treatment as described in 2.2. As shown previously, *Klotho* knock down leads to significant decrease of the percentage of cells expressing Klotho by -17.2±1.4%, $p<0.001$ (Figure 5-3a) at 3-day proliferation and significantly decreases the percentage of Klotho expressing cells -67.3±0.5%, $p<0.001$(Figure 5-3e) at 7-day differentiation, confirming that the *Klotho* knock down successfully decreases the proportion of Klotho expressing cells in proliferating and differentiating HPC03A/07.

5.4.1.1 RSVL impacts on Klotho expression despite a Klotho knock down

Figure 5-3c shows that during proliferation RSVL treatment following *Klotho* knock down significantly increases the percentage of Klotho positive cells compared to *Klotho* knock down alone (73.6±0.2%, $p<0.001$); however the percentage is significantly lower by -34.6±0.0%, $p<0.01$ compared to RSVL only treated cultures (Figure 5-3b). This suggests that RSVL might increase *Klotho* mRNA to such an extent that the amount of siRNA is not sufficient to bind to all *Klotho* mRNA leading to Klotho protein being produced even in cells transfected with *Klotho* targeting siRNA. Under these circumstances it would be interesting to conduct a dosage experiment and titrate the siRNA concentration needed to fully block the RSVL induced *Klotho* mRNA activation. The specific PPARγ agonist rosiglitazone has no impact on the number of Klotho positive cells (Figure 5-3c) after *Klotho* knock down. This indicates that PPARγ activation has no impact on *Klotho* mRNA during *Klotho* knock down during proliferation

On the other hand, data presented in Figure 5-3g shows that during differentiation conditions specific PPARγ activation (94.5±8.1%, $p<0.001$) and RSVL treatment (145.5±11.3, $p<0.001$) both significantly increase the percentage of Klotho positive cells following *Klotho* knock down compared to the *Klotho* knock down without following treatment; however the proportions are significantly decreased compared to the RSVL only group by -45.7±2.5%, $p<0.001$ (Figure 5-3f). This suggests that in differentiating HPC03A/07 cultures PPARγ activation and RSVL treatment impact on *Klotho* expression despite a >80% knock down.

5.4.1.2 RSVL partly requires Klotho to increase the number of Ki67 expressing cells in proliferating but not differentiating HPC03A/07

Klotho knock down significantly decreases (-14.9±6.8%, $p<0.05$) the proportion of Ki67 positive cells compared to DMSO treated control cultures during proliferation (Figure 5-4a). In differentiating cultures RSVL has no effect on the proportion of dividing cells.

Klotho appears to be partly required under RSVL treatment to increase the number of dividing cells to the level of RSVL only treatment. Indeed, upon RSVL treatment on *Klotho* knock down cells, the percentage of Ki67 expressing cells is significantly decreased by -11.4±1.5%, $p<0.01$ compared to RSVL only, see Figure 5-4b. On the other hand, data presented in Figure 5-4c shows that PPARγ activation, but not RSVL, significantly increases the percentage of Ki67 positive cells following *Klotho* knock down when compared to the *Klotho* knock down only group (Rosiglitazone: 25.9±4.5%, $p<0.05$). This suggests that PPARγ activation does not require *Klotho* to increase the proportion of dividing cells to the level of the vehicle control, but it is necessary under RSVL treatment to further increase the proportion of dividing cells to the RSVL only level under proliferative conditions.

Under differentiation conditions Figure 5-4g shows that PPARγ activation (96.6±14.9%, $p<0.001$) and RSVL treatment (115.3±1.2, $p<0.001$) both significantly increase the percentage of Ki67 positive cells following *Klotho* knock down compared to the *Klotho* knock down alone; even to the level of the RSVL only group, (Figure 5-4f). This indicates that neither RSVL nor Rosiglitazone, the specific PPARγ agonist require *Klotho* to impact on the proportion of dividing cells under differentiation conditions.

5.4.1.3 RSVL requires both Klotho and PPARγ to increase neurogenesis in differentiating HPC03A/07

Klotho knock down decreases the percentage of Dcx (by -22.8±0.3%) and MAP2 (-8.9±0.4%, $p<0.05$) expression significantly compared to DMSO treated cultures (Figure 5-5a,e).

The percentage of Dcx and MAP2 positive cells is significantly decreased after RSVL treatment following *Klotho* knock down by -47.5±0.8%, $p<0.001$ and -40.9±1.1%, $p<0.001$ respectively compared to the RSVL only treated cells, suggesting that *Klotho* is needed for RSVL to elicit its effect on neurogenesis (Figure 5-5b,f)

In turn PPARγ activation (17.8±7.4%, $p<0.05$) and RSVL treatment (27.0±1.9%, $p<0.05$) following *Klotho* knock down both significantly increase the percentage of Dcx positive cells compared to the *Klotho* knock down alone, see Figure 5-5c; suggesting that *Klotho* is required to increase the percentage of neuroblasts to the level of RSVL but not to increase it above the *Klotho* knock down only.

On the other hand, Rosiglitazone, the specific PPARγ agonist does not have the ability to increase MAP2 expression without *Klotho* and even decreases the percentage of MAP2 positive cells by -25.8±1.1%, $p<0.001$, Figure 5-5g. RSVL treatment however significantly increases the percentage of MAP2 positive cells following *Klotho* knock down compared to the *Klotho* knock down alone by 27.8±2.3%, $p<0.001$). This indicates that RSVL does not require *Klotho* and PPARγ to increase the proportion of mature neurons above vehicle level, however they are necessary to reach the level of RSVL only treated cells.

Further, PPARγ cannot impact on MAP2 without *Klotho*; this is in contrast to the effect of PPARγ on Dcx which does not depend on *Klotho*.

5.4.1.4 RSVL decreases the Klotho knock down induced apoptosis in differentiating HPC03A/07

I first confirmed in this independent set of experiments what I discovered in 3.2.3 RSVL treatment significantly increases the percentage of activated Caspase-3 expressing cells by 24.2±0.9%. *Klotho* knock down alone increases the percentage of apoptotic cells by 96.1±0.7%, indicating that both RSVL and *Klotho* knock down increase apoptosis in differentiating HPC03A/07 cells.

Klotho knock down in differentiating HPC03A/07 followed by RSVL treatment increases the percentage of apoptotic cells compared to RSVL only treatment by 22.5±1.4%, whereas when PPARγ is blocked before RSVL treatment apoptosis is decreased by -15.6±0.3% (Figure 5-6b). However, when *Klotho* is knocked down prior to RSVL treatment the percentage of apoptotic cells is decreased compared to the *Klotho* knock down only cultures by -44.8±3.4% and also when *Klotho* knock down is followed by PPARγ activation apoptosis is decreased by -61.5±0.8% (Figure 5-6a). This suggests that RSVL treatment, although increasing apoptosis on its own has the ability to decrease apoptosis following *Klotho* knock down.

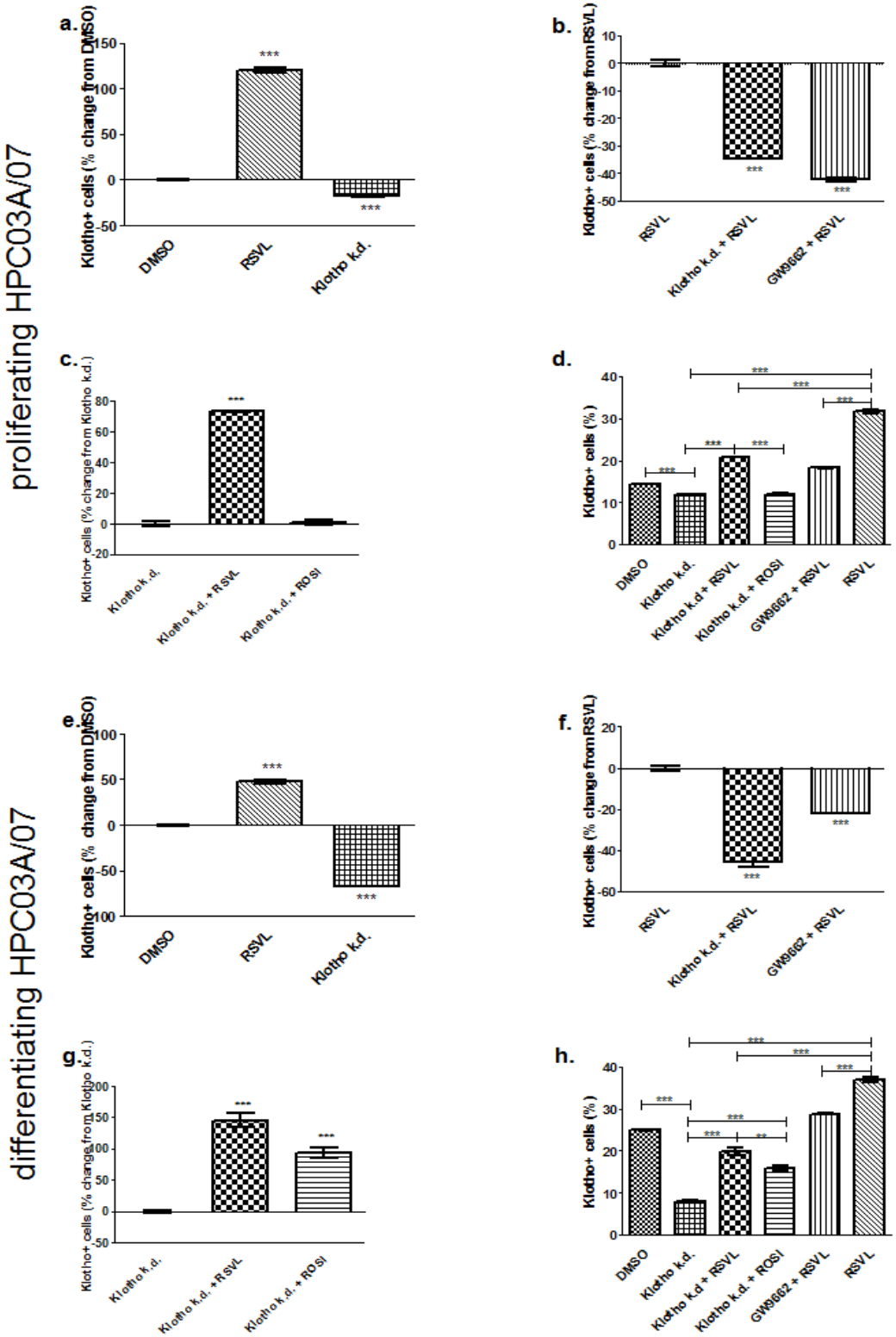


Figure 5-3 Role of PPARγ and Klotho for Klotho activation through RSVL in proliferating and differentiating HPC03A/07

a, b, c, d depict proliferating e, f, g, h differentiating cultures. Percentage change of Klotho positive cells in treated cultures compared to (a and e) DMSO treated cultures, b) and f) RSVL treated cultures, c) and g) Klotho knock down cultures. A Summary of the quantification of Klotho positive cells (absolute numbers) is shown in (d and h).DMSO: vehicle; RSVL: Resveratrol; ROSI: PPARγ agonist Rosiglitazone; GW9662: PPARγ antagonist; Klotho k.d.: Klotho knock down (p-values were generated using One-way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), ***p<0.001)

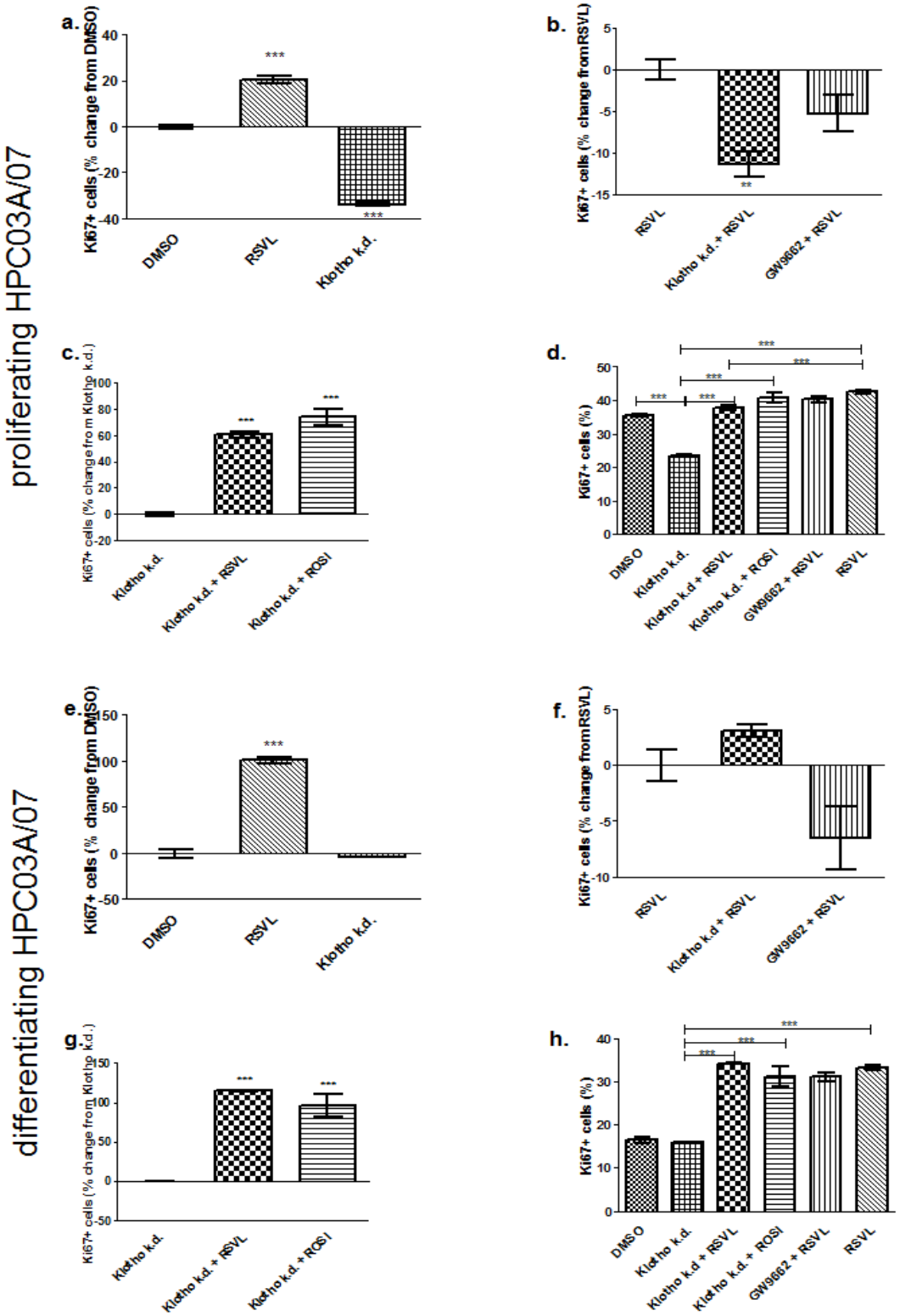


Figure 5-4 Impact of PPARγ and Klotho the percentage of Ki67 cells through RSVL in proliferating and differentiating HPC03A/07

a, b, c, d depict proliferating e, f, g, h differentiating cultures. Percentage change of Ki67 positive cells in treated cultures compared to (a and e) DMSO treated cultures, (b and f) RSVL treated cultures, (c and g) Klotho knock down cultures. A Summary of the quantification of Ki67 cells (absolute numbers) is shown in (d and h).DMSO: vehicle; RSVL: Resveratrol; Klotho k.d.: Klotho knock down; ROSI: PPARγ agonist Rosiglitazone; GW9662: PPARγ antagonist; (p-values were generated using One-way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), ***p<0.001)

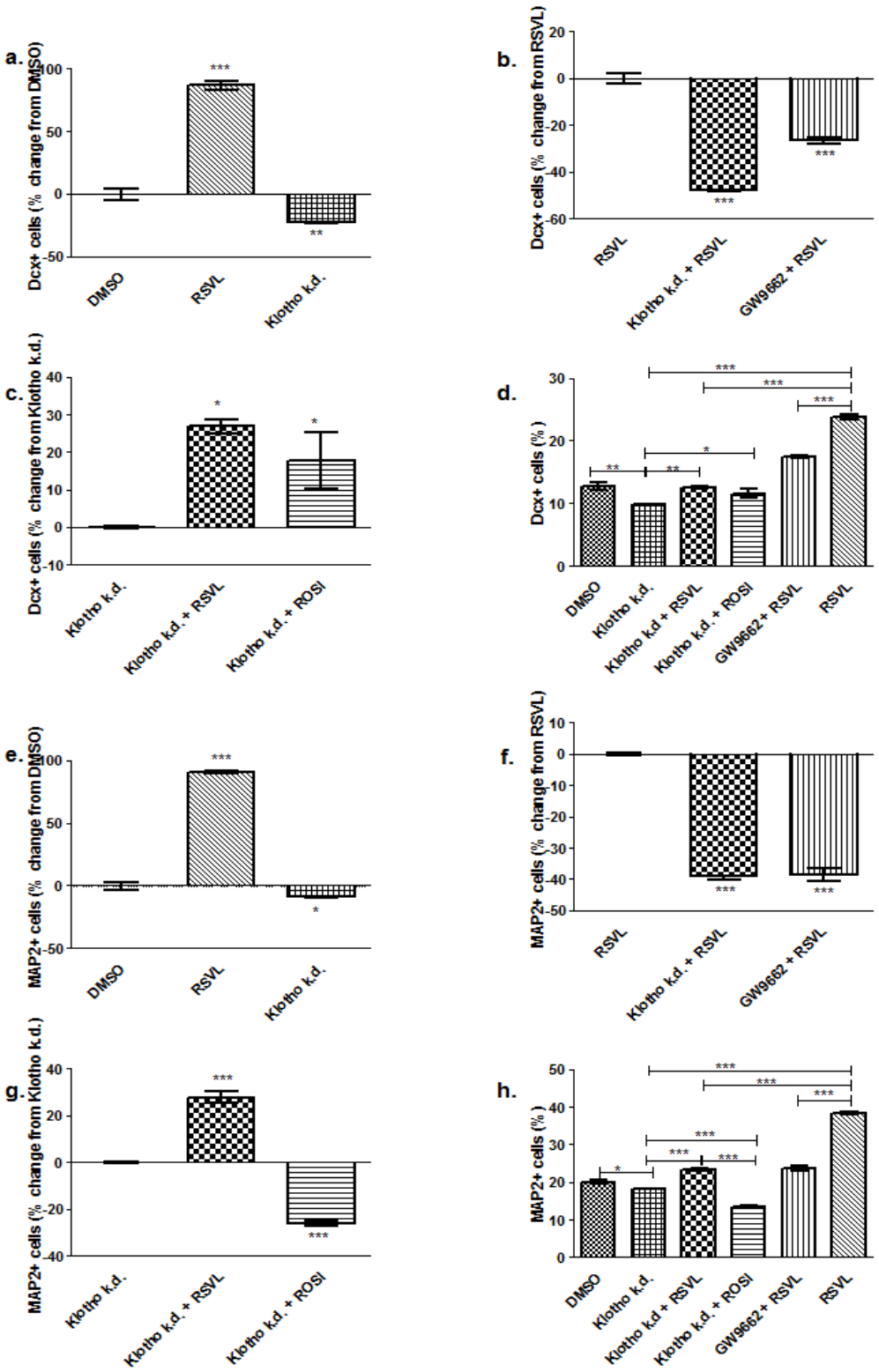


Figure 5-5 Role of PPARγ and Klotho for Dcx and MAP2 expression through RSVL in differentiating HPC03A/07

a, b, c, d depict a quantification of Dcx; e, f, g, h of MAP2. Percentage change of Dcx/MAP2 positive cells in treated cultures compared to (a and e) DMSO treated cultures, (b and f) RSVL treated cultures, (c and g) Klotho knock down cultures. A Summary of the quantification of Dcx/MPA2 positive cells (absolute numbers) is shown in (d and h). DMSO: vehicle; RSVL: Resveratrol; Klotho k.d.: Klotho knock down; ROSI: PPARγ agonist Rosiglitazone; GW9662: PPARγ antagonist; (p-values were generated using One-way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, **p<0.01, ***p<0.001)

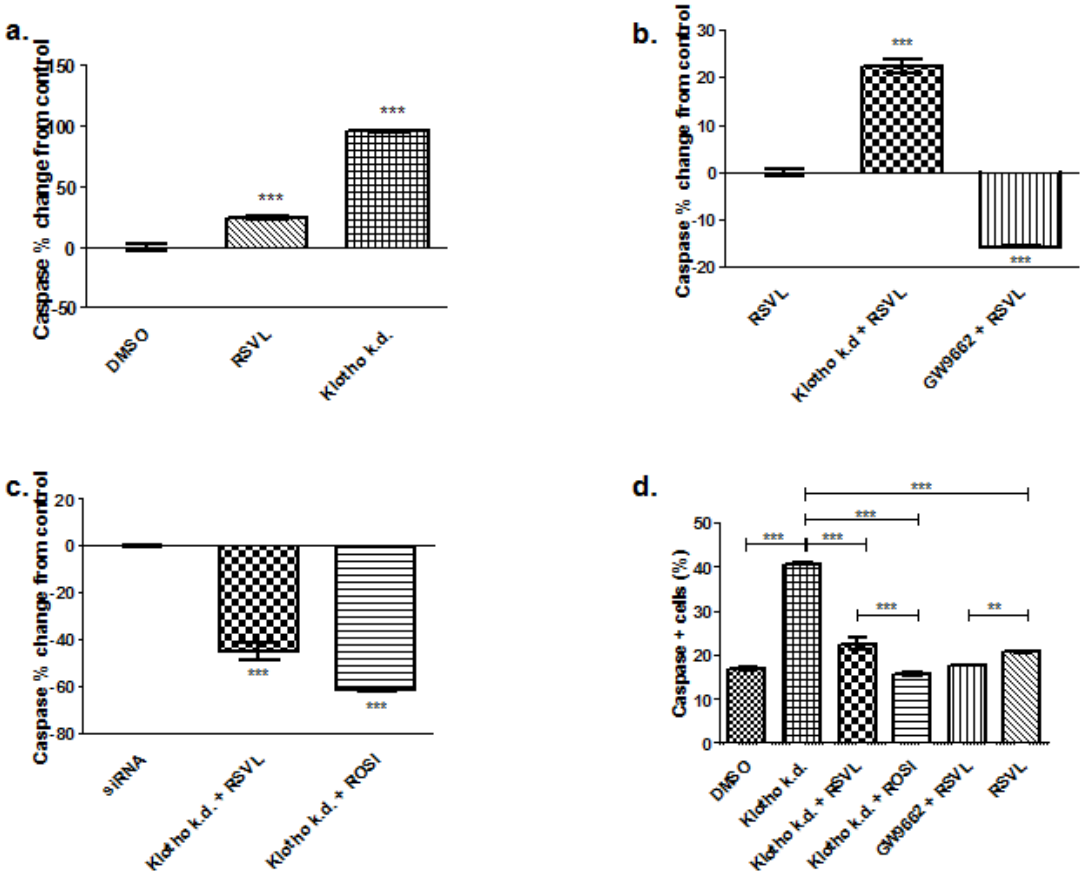


Figure 5-6 Role of PPARγ and Klotho for activated Caspase-3 activation through RSVL in differentiating HPC03A/07

Percentage change of activated Caspse-3 positive cells in treated cultures compared to a) DMSO treated cultures, b) RSVL treated cultures, c) Klotho knock down cultures. A Summary of the quantification of Klotho positive cells (absolute numbers) is shown in (d). DMSO: vehicle; RSVL: Resveratrol; Klotho k.d.: Klotho knock down; ROSI: PPARγ agonist Rosiglitazone; GW9662: PPARγ antagonist; (p-values were generated using One-way ANOVA with Newman-Keuls post hoc test, Error bars are the Standard Error of the Mean (SEM), *p<0.05, ***p<0.001,)

5.5 Summary

To summarize, an overview of the quantification of Klotho positive cells during proliferation and differentiation after being treated as described in Figure 2-10 and Figure 5-2 is shown in Table 5-1. It confirms the successful *Klotho* knock down and demonstrates that RSVL partly requires PPARγ for *Klotho* activation during proliferation and neurogenesis in HPC03A/07 cells and acts on *Klotho* despite a >80% *Klotho* knock down. RSVL might increase *Klotho* mRNA to such an extent that the amount of siRNA is not sufficient to bind to all *Klotho* mRNA leading to Klotho protein being produced in cells after *Klotho* knock down.

Proliferation:

RSVL treatment increases the percentage of Klotho expressing cells compared to the *Klotho* knock down cultures after PPARγ block and *Klotho* knock down during proliferation. However never to the level of RSVL only treated cultures, suggesting that PPARγ is only partly involved in the effect RSVL has on *Klotho*. *Klotho* expression appears to be higher during differentiation than proliferation in all treatment groups and during proliferation PPARγ activation has no effect on Klotho compared to the *Klotho* knock down cultures. Altogether, this data indicates that RSVL is very potently activating *Klotho* expression and is also independent of a PPARγ dependent pathway.

Figure 5-4d shows a summary of the quantification of Ki67 positive cells after being treated as described in Figure 2-10 and Figure 5-2. RSVL partly requires *Klotho* but does not depend on PPARγ to increase the proportion of Ki67 expressing cells in proliferating and PPARγ does not require *Klotho* to increase the proportion of dividing cells. Altogether, this set of data shows that PPARγ is not required for RSVL to act on dividing cells and neither RSVL nor PPARγ

activation do require *Klotho* to increase the proportion of dividing cells compared to control conditions, however I also demonstrate here that RSVL partly requires *Klotho* to fully increase the proportion of Ki67 positive cells to the level observed under RSVL treatment.

Differentiation:

RSVL treatment increases the percentage of Klotho expressing cells compared to the *Klotho* knock down cultures after PPARγ block and *Klotho* knock down during differentiation. However never to the level of RSVL only treated cultures, suggesting that PPARγ is only partly involved in the effect RSVL has on *Klotho*. *Klotho* expression appears to be higher during differentiation than proliferation in all treatment groups and during differentiation PPARγ activation increases Klotho despite the knock down. Altogether, this data indicates that RSVL is very potently activating *Klotho* expression and is also independent of a PPARγ dependent pathway.

Figure 5-4h shows a summary of the quantification of Ki67 positive cells during differntiation. RSVL does not require *Klotho* or PPARγ to increase the proportion of Ki67 expressing cells and *Klotho* is not required for PPARγ to increase the proportion of dividing cells in differentiating HPC03A/07. Altogether, this set of data shows that RSVL does not require PPARγ and neither RSVL nor PPARγ activation do require *Klotho* to increase the proportion of dividing cells compared to control conditions, however I also demonstrate that RSVL partly requires *Klotho* to fully increase the proportion of Ki67 positive cells to the level observed under RSVL treatment.

RSVL partly requires both *Klotho* and PPARγ to increase neurogenesis in differentiating HPC03A/07. Figure 5-5d shows a summary of the quantification of Dcx positive cells. RSVL does not require *Klotho* and PPARγ to increase the proportion of Dcx positive cells compared to the control; however both are necessary to increase Dcx to the full level of RSVL only. Further, PPARγ activation partly requires *Klotho* to impact on Dcx expression. On the other hand the summary of the quantification of MAP2 (Figure 5-5h) illustrate my findings on the ability of RSVL but not of the specific PPARγ agonist Rosiglitazone to activate differentiation independently of *Klotho*. Furthermore, it shows that PPARγ and *Klotho* are required for RSVL to increase MAP2 expression to its full potential.

Figure 5-6d shows a summary of the quantification of activated Caspase-3 expressing cells in differentiating HPC03A/07. Absence of PPARγ prevents RSVL induced apoptosis, while on the other hand RSVL and PPARγ activation decrease the *Klotho* knock down induced apoptosis in differentiating HPC03A/07. This indicates that RSVL treatment increases survival following *Klotho* knock down suggesting that RSVL during stress prevents cell death although RSVL on its own increases apoptosis, PPARγ and *Klotho* both appear to be involved in apoptosis in HPC03/A07 cells.

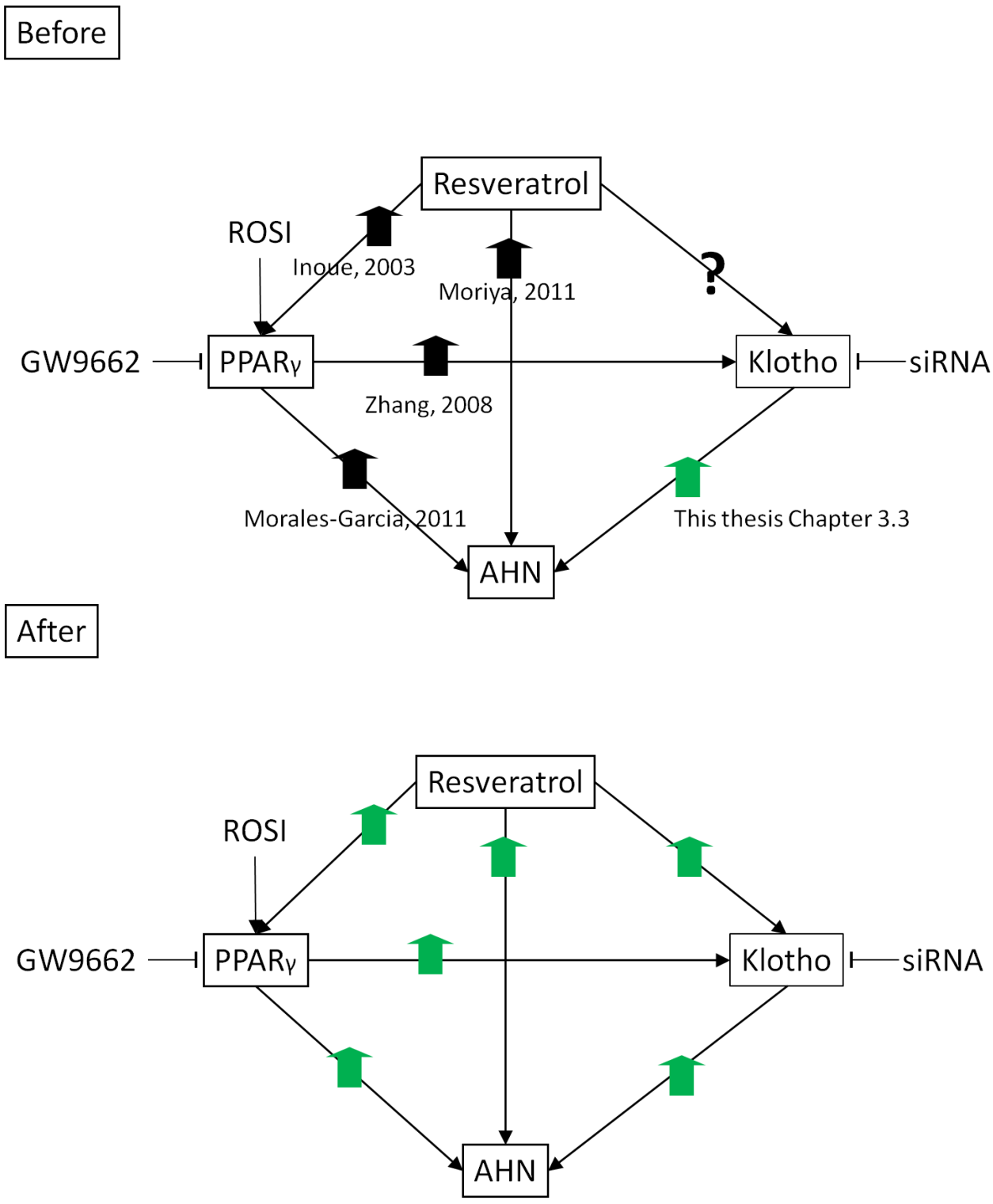


Figure 5-7 Effect of Resveratrol on AHN, Klotho and PPARγ

The graph shows what was known in the literatur before his thesis and after. RSVL impacts on AHN via PPARγ and Klotho, but also directly via an independent pathway. PPARγ and Klotho are both partly required for RSVL to exert its effect on AHN to its full potential.

Abbreviations:AHN: adult hippocampal neurogensis; RSVL: Resveratrol; ROSI: the PPARγ activator Rosiglitazone; GW9662: PPARγ blocker; black arrows indicate the effet shown in the literature; green arrows indicate the effect shown in the thesis in HPC03A/07

	RSVL w/o Klotho		**ROSI w/o Klotho**		**RSVL w/o PPARγ**	
Compared to:	KL k.d.	RSVL	KL k.d.	RSVL	DMSO	RSVL
Proliferation						
Klotho	↑	↓	-	↓	-	↓
Ki67	↑	↓	↑	-	↑	-
Differentiation						
Klotho	↑	↓	↑	↓	↑	↓
Ki67	↑	-	↑	-	↑	-
Dcx	↑	↓	↑	↓	↑	↓
MAP2	↑	↓	↓	↓	↑	↓
Activated Caspase-3	↓	↑	↓	↓	-	↓

Table 5-1 Summary of the effect of RSVL after *Klotho* knock down and PPARγ block.

↑ significant increase; ↓ significant decrease; - no significant changes RSVL: Resveratrol; Kl k.d.: *Klotho* knock down; ROSI: Rosiglitazone

5.6 Discussion

5.6.1 PPARγ is partly required for RSVL stimulated Klotho expression and neurogenesis, but not for proliferation

Firstly my results proved that RSVL increases the number of *Klotho* expressing cells in proliferating and differentiating HPC03A/07 cultures. My results further show that RSVL partly requires PPARγ to increase the number of *Klotho* expressing cells during proliferation as well as during differentiation. RSVL impacts on the proportion of Klotho positive cells despite a >80% *Klotho* knock down. This suggests that RSVL might increase *Klotho* mRNA to such an extent that the amount of siRNA is not sufficient to bind to *Klotho* mRNA leading to Klotho protein being produced even in cells transfected with *Klotho* targeting siRNA. Under these circumstances it would be interesting to titrate the siRNA concentration needed to fully block the RSVL induced *Klotho* mRNA activation.

These results are in line with Zhang et al. that demonstrated that a PPARγ agonist increases *Klotho* expression in mouse kidneys whereas PPARγ antagonists reduce its expression. However, I am the first to demonstrate PPARγ is partly required for RSVL to elicit its *Klotho* increasing action. In my results RSVL was still able to increase the proportion of Klotho expressing cells despite PPARγ being blocked and even despite a previous *Klotho* knock down, indicating that RSVL acts on *Klotho* expression not only via PPARγ but also via an alternative, maybe direct pathway. RSVL acts on various targets that are associated with the protection against aging related diseases, such as diabetes and cardiovascular diseases. RSVL acts on SIRT1 that has been associated with aging and the nuclear transcription factors PPARα, δ and γ that are involved in cell cycle mechanisms. Also COX1 and 2, enzymes involved in

inflammatory processes and eNOS that regulates vascular tone by generating nitric oxide (NO) synthase that generates NO in blood vessels (Nakata et al., 2012). This demonstrates that RSVL most likely acts on organisms via many pathways.

My results also show that RSVL partly requires *Klotho* but not PPARγ to act on dividing cells; moreover RSVL increases the proportion of dividing cells in differentiating cultures; however this action partly requires *Klotho*, only during proliferation, but not PPARγ. RSVL increases the proportion of Ki67 positive cells almost to the level of RSVL only treated cultures despite a *Klotho* knock down during proliferation. During differentiation neither *Klotho* nor PPARγ are required for RSVL to act on dividing cells. This suggests that RSVL actually requires *Klotho* and/or PPARγ to impact on proliferation not at all or to a minimal extent. This is in accordance with my data on Klotho (Section 0 and 4.4.4), where neither *Klotho* over-expression nor a *Klotho* knock down significantly affected cell division, suggesting that RSVL and *Klotho* target different pathways.

Although RSVL has dose and duration dependent chemopreventive, proapoptotic, antiproliferation and anti-inflammation effects in cancer cells (Aluyen et al., 2012), its positive effects on healthy organisms are widely accepted (Nakata et al., 2012). However, it is not understood how one molecule can have such dramatically differently effects. RSVL not only reduces brain injury but also promotes recovery after stroke by promoting proliferation and migration. It also activates phosphoinositide 3 kinase (PI3-K)/Akt and Mitogen-Activated Protein Kinase (MAPK)/ERK signalling pathways that are involved in cellular activities such as differentiation and apoptosis in a human cerebral

endothelial cell line (Simao et al., 2012) which could explain how RSVL in my results increases differentiation and apoptosis. Interestingly, in one study conducted in rats with and without diabetes, RSVL could not increase proliferation in the hippocampus of nondiabetic rats whereas in diabetic animals it could (Venturini et al., 2010). This indicates that RSVL might only unfolds its potential in influencing cell cycle mechanism under systemic stress, as I have also established in the Cortisol stress model, where RSVL, despite increasing apoptosis on its own, can prevent the stress mimicking Cortisol induced effects on dividing cells, differentiation and apoptosis. Further supporting this idea are my data that show that RSVL treatment decreases the apoptosis induced by *Klotho* knock down.

RSVL does not require *Klotho* and PPARγ activation to increase the proportion of Dcx positive cells. However both are necessary to increase Dcx to the full level seen in differentiating cultures treated with RSVL alone. On the other hand, PPARγ requires *Klotho* to increase Dcx expression supporting the conclusion that RSVL can impact on neurogenesis independently of PPARγ and directly via *Klotho* or even independently of *Klotho*. However, PPARγ and *Klotho* are required for RSVL to increase the proportion of mature neurons to the level of RSVL only treated cultures. Morales-Garcia recently demonstrated that the PPARγ activator Rosiglitazone regulates neural stem cell proliferation and differentiation both *in vivo* and in primary cultures in form of an increase in Dcx positive cells (Morales-Garcia et al., 2011). My data shows that Rosiglitazone however requires *Klotho* to increase neurogenesis in HPC03A/07 cells as it was not able to increase the percentage of Dcx and MAP2 positive cells after *Klotho* knock down.

Interestingly, in a recent study by Bruedigam et al. Rosiglitazone and RSVL were shown to have opposing effects in vascular smooth muscle cells (VSMC) on vascular mineralisation (Bruedigam et al., 2011). Rosiglitazone stimulates mineralisation partly via caspase-dependent apoptosis and induces oxidative stress, which leads to an osteoblast-like differentiation phenotype. RSVL in turn reduces the osteoblast-like differentiation as well as the pro-apoptotic and pro-calcifying effects of rosiglitazone. It has been reported repeatedly that rosiglitazone, used as an insulin sensitizer to treat type-II diabetes mellitus , is also associated with an increased risk of heart attacks (Nissen and Wolski, 2007). This also suggests that RSVL acts not only as a PPARγ. However, I have analysed the percentage of activated Caspase-3 expressing cells as part of my study to see the effect of apoptosis after rosiglitazone treatment and could see no significant differences to the vehicle when cultures were treated with rosiglitazone after *Klotho* knock down, moreover rosiglitazone significantly decreased the percentage of apoptotic cells compared to the *Klotho* knock down only treatment suggesting PPARγ activation, similar to RSVL, rescues the increasing effect *Klotho* knock down has on apoptosis. My data showed that RSVL treatment, although increasing apoptosis on its own, has the ability to decrease apoptosis following *Klotho* knock down. PPARγ activation by Rosiglitazone and RSVL treatment decrease the percentage of apoptotic cells to the level of the vehicle control. This indicates that RSVL treatment increases survival in HPC03A/07 cells following *Klotho* knock down and PPARγ block. These differences in cell death might be explained by the different cell culture system. Bruedigam et al used VSMC and rosiglitazone at a concentration of 10µM whereas I have used HPC03A/07 and rosiglitazone at 3µM. Next it would be interesting to analyse the effect of rosiglitazone alone on proliferation,

differentiation and apoptosis in HPC03A/07. PPARγ activation following a *Klotho* knock down did not increase the proportion of Klotho, Dcx and MAP2 positive cells, however it increased the proportion of dividing cells and decreased apoptosis following *Klotho* knock down. Compared to this, in HPC03A/07 cells RSVL has the ability to increase *Klotho* expression, proliferation and neurogenesis even after a *Klotho* or PPARγ knock down or block respectively. Another reason for a RSVL effect beyond PPARγ is that it also impacts not only on PPARγ but also on PPARα and δ in BAECs (Inoue et al., 2003; Tsukamoto et al., 2010).

In short lived organisms such as fruit flies and nematodes, RSVL increases life span (Wood et al., 2004) and in yeast RSVL feeding increases life span and also the activity of the enzyme Sir2. Sir2, in mammals SIRT1, plays a key role in an organism's response to stresses, is associated with longevity and is also increased during caloric restriction (CR) (Lin et al., 2000; Howitz et al., 2003).

Also *Klotho* has been shown to increase lifespan in mice, however most likely by inhibiting insulin/IGF1R signalling (Kurosu et al., 2005). Negative regulation of insulin/IGF1R signalling is an evolutionarily conserved mechanism to suppress aging and extends life span in yeast, nematodes, fruit fly and rodents (Barbieri et al., 2003; Tatar et al., 2003). On the other hand endogenous IGF1 plays an important role for and neurogenesis in the adult rat brain development (Anderson et al., 2002; Koltai et al., 2011) and during brain development in human (Netchine et al., 2011).

Barger et al compared the effects on gene expression of caloric restriction (CR) (approximately 10% reduction compared to ad libitum fed mice) with RSVL supplementation (50mg/kg diet, 4.9mg/g body weight) from middle to old age in

mice. RSVL mimics CR at the gene expression level, but did not mimic the effects of CR on the few proteins examined (amongst those were IGF1 and SIRT1). These differences might be due to differences at the translational regulation (Barger et al., 2008b). On gene expression level RSVL, unlike CR did not reduce circulating IGF1 levels. Also both CR and RSVL do not alter SIRT1 levels and CR but not RSVL induces PGC-1α transcriptional targets (Barger et al., 2008b). Based on these results it is unlikely that low doses RSVL supplementation exerts its beneficial effects on lifespan and health via a SIRT1 or IGF1 involving pathway. Also Chang et al. showed that a RSVL supplemented diet did not significantly improve radial arm water maze function, did not increase sirtuin 1 (SIRT1) expression or downstream marker p53 of sirtuin 1 activation. Further markers of cellular stress, inflammation, and AD pathology were not modulated by RSVL. Chang et al used SAMP8 mice, a model of accelerated aging that is increasingly being validated as a model of sporadic and age-related AD, and RSVL at by diet achievable concentrations (explained below). The RSVL concentration (120 mg/kg diet) was equivalent to the content of RSVL in two glasses of wine. (Chang et al., 2011).

Recently cAMP signalling as an alternative pathway has been shown to be a target of RSVL by suppressing the enzyme phosphodiesterase (PDE) (Park et al., 2012). cAMP signalling is highly complex and is a key mediator of metabolic regulation. The identification of PDEs as RSVL targets might explain how RSVL mimics some aspects of CR. CR increases cAMP levels by increasing glucagon and catecholamine signalling and decreasing insulin/IGF-1 signalling (Rondinone et al., 2000). PDE4 inhibitors have been shown to improve memory (Burgin et al., 2010) and protection against aging-related diseases such as Alzheimer's (Smith et al., 2009) animal models. It is therefore possible that

RSVL acting as a PDE4 inhibitor may be useful for treating systemic and age related diseases humans.

Chapter 6 Final Discussion and Conclusion

6.1 Summary of findings

Using a model *in vitro* system based on human foetal hippocampal progenitor cells (HPC03A/07 cells) I investigated the cellular and molecular mechanisms that underlie the effects of certain dietary components on the proliferation and neuronal differentiation of human hippocampal progenitor cells. Specifically, I investigated the direct effects of the Omega-3 fatty acids EPA and DHA and the stilbenoid Resveratrol on human hippocampal NPC proliferation and neurogenesis, as well as their potential in preventing the negative effects of high Cortisol concentrations (100µM) on proliferation, differentiation and apoptosis (0). I further investigated the role of *Klotho* and the nuclear transcription factor PPARγ on human hippocampal NPC proliferation and neurogenesis. The main findings of my research are summarised below:

1. The Omega-3 fatty acids EPA and DHA primarily promote survival resulting in an increased proportion of dividing progenitor cells and hence an increase in the proportion of neurons being generated and also increases neurogenesis.
2. The effects of the stilbenoid Resveratrol are concentration dependent: It leads to cell death at very high concentrations (10µM), decreases the proportion of dividing cells at high concentrations (5µM) due to increased apoptosis. Lower concentrations (1µM) increase the proportion of dividing cells which seems based on an increase in proliferation as apoptosis was not decreased.
3. In an *in vitro* model of stress, pre-treatment with EPA and DHA prevents the negative effect of high concentrations of Cortisol (100µM) on proliferation,

neurogenesis and apoptosis by promoting survival and enhancing neurogenesis.

4. RSVL pre-treatment in an *in vitro* model of stress prevents the negative effects of high, stress-relevant concentrations of Cortisol (100µM) on proliferation and neurogenesis, but not on apoptosis by promoting neurogenesis and cell division.
5. In wild type mouse brain, Klotho protein is expressed in neurons (NEUN) and stem cells (SOX2), but not in glial cells (GFAP).
6. Intermittent fasting does not increase the total proportion of Klotho expressing cells in the dentate gyrus of mice, but the existing Klotho positive cells produce more Klotho protein.
7. Over-expression of *Klotho* increases neurogenesis and apoptosis in HPC03A/07 during differentiation. This effect appears to be due to enhanced neurogenesis as *Klotho* seems to be involved in fate commitment, neurogenesis and neuronal survival.
8. *Klotho* knock-down shows that *Klotho* is required for neurogenesis and survival during differentiation, but is not important for dividing cells.
9. *Klotho* is not strictly required for RSVL to exert its positive effect on proliferation and differentiation. RSVL modulates the proportion of dividing cells and neurogenesis via a *Klotho* dependent pathway as well as via a *Klotho* independent pathway.
10. RSVL increases proliferation and neurogenesis even after PPARγ inactivation and increases *Klotho* expression only during differentiation. This suggests that RSVL acts on proliferation and neurogenesis via a PPARγ dependent as well as a PPARγ independent pathway. It further indicates that the impact on differentiation of RSVL via *Klotho* is more prominent.

6.2 Methodological considerations

There are a few methodological limitations that need to be considered when interpreting the findings obtained in this PhD thesis.

6.2.1 The use of *in vitro* systems to study human hippocampal neurogenesis

In this thesis I have used a conditionally immortalised human foetal hippocampal progenitor cell line (HPC03A/07) to examine the effects of dietary components on hippocampal neurogenesis.

The major disadvantage of *in vitro* experimental studies is that they fail to replicate the precise cellular environmental conditions of an organism. *In vivo*, progenitor cells are never alone. Their relationship to a neurogenic microenvironment might be inseparable from their inherent properties. In principle the conditions *in vitro* are artificial and are approximate reconstitutions of biological processes by mixing the necessary components and reagents under controlled conditions, stressing the utmost importance of understanding the *in vivo* microenvironment in which neural precursors reside. Therefore it can be very difficult to extrapolate from the results of *in vitro* work back to the biology of the intact organism. Care must be taken not to over-interpretation *in vitro* results, which can sometimes lead to incorrect conclusions about the biology of the organism and the system. However, there are currently no other tools available to investigate the molecular mechanisms that underlie hippocampal neurogenesis in humans, and all studies aiming to elucidate the signalling mechanisms of dietary components on neurogenesis have so far only been conducted in animals or animal tissue culture. Although animal models do indeed allow for an investigation of a whole organism, apparent differences

between rodents and humans exist in physiology and behaviour. And translating human depressive pathophysiology into rodent behavioural paradigms of depression is especially a great challenge.

On the other hand the simplicity of *in vitro* systems is a great advantage as it allows to examining the contribution of specific factors, such as dietary components or drugs, without confounding influences, which cannot be eliminated in *in vivo* models, but simply added or omitted in a cell culture system. Moreover investigating the effects of these factors on specific cell types enables the researcher to disentangle cell-type specific properties of certain treatments from their effects on more complex brain systems.

The changes observed in this human hippocampal progenitor cell line *in vitro* regarding the effects of Omega-3 fatty acids, RSVL and Klotho are consistent with *in vivo* findings in the literature to date, which further validates the use of the cell line as a research tool to investigate molecular mechanisms in human hippocampal neurogenesis.

6.2.2 The relevance of foetal progenitor cells

The cell line used in this thesis was a foetal human hippocampal progenitor cell line. Although these cells have the advantage of being human, their properties could potentially differ from the ones of hippocampal progenitor cells in the adult brain. Nevertheless, these cells express the same markers as adult hippocampal progenitor cells and they also have the same properties to proliferate and to differentiate into neurons and glia (as described in 2.1.1). Although working with adult human brain cells would be the technically preferred system, however for obvious ethical reasons this is impossible. While hippocampal progenitor cells can be isolated from adult rodent brains, apparent

species-specific differences in physiology and behaviour also appear in the genetics, despite being very similar to the human genome. Therefore for this thesis the human hippocampal progenitor cell line was chosen to investigate the molecular mechanism of dietary components.

6.2.3 The use of dietary components *in vitro*

When testing the effect of dietary components on the brain in an *in vitro* system in order to be later used *in vivo* primary considerations are, whether they can reach the brain, cross the blood brain barrier as well as their metabolism on their way through the digestive system and the circulation. Firstly, EPA, DHA and RSVL can cross the blood barrier and thereby reach the brain. Dietary DHA is well absorb and is readily incorporated into the plasma, blood cell lipids (Innis, 1991). Several animal studies have also shown that dietary DHA is readily incorporated into lipids of the developing brain, both before and after birth (Innis, 2005). *in vitro*, glia and cerebral endothelial cells, but not neurons, can form DHA from ALA and other precursor Omega-3 FAs (Moore, 2001). A conversion of EPA to DHA in the HPC03A/07 cell line is possible as we have checked that the cells express the genes for the required enzymes *in vivo*. However it is not clear what exactly mediates the effect; It is most likely that the effect is mediated by EPA and DHA directly, however oxidised metabolites and eicosanoids also have to be considered.

RSVL however is rapidly metabolised after oral uptake into conjugated forms such as glucuronate and sulfonate, which makes its oral bioavailability very low (Walle et al., 2004). After an oral dose of 25mg only trace amounts of RSVL can be detected in the blood; however plasma concentrations of RSVL and metabolites peaked at concentrations around 2 µM 1h later (Walle et al., 2004).

The concentration used in this study was with 1µM even lower and still had an effect on cell division and differentiation, which confirms that in this thesis physiological achievable concentration of RSVL were used. Investigating the effects of RSVL in a tissue culture system on cell cycle mechanism is therefore a valid system; however bioavailability and its metabolites should be examined *in vivo*.

There are further issues with recommending concentrations for RSVL as a supplement as most of the beneficial studies highlighted earlier actually used concentrations higher than achievable by a normal diet. So what are by diet achievable concentrations *in vivo*? In a human study that addressed absorption and bioavailability of RSVL, serum concentrations only reach 20-50nM after oral administration of 25mg in healthy humans (Goldberg et al., 2003; Walle et al., 2004). 25mg of RSVL are approximately contained in 2.3l of red wine (Pinot noir), 16.6kg of raw peanuts or 20.2kg of dark chocolate. Compared to this two glasses (150ml) of Pinot noir contain 3.14mg of RSVL (Chachay et al., 2011):

All together the literature on RSVL is contradictory and confusing, due to the great variability of concentrations and doses used to achieve various effects in different *in vitro* and *in vivo* models and systems. Furthermore, RSVL is highly rapidly and extensively metabolised in the body, resulting in little unchanged RSVL in the systemic circulation, even at fairly high concentration of RSVL RSVL after the 25-mg oral dose (Walle et al., 2004). This suggests that further research is needed into the metabolic effects of the RSVL metabolites have on cellular mechanisms.

Nonetheless, beneficial effects of plants rich in RSVL such as grapes have been reported for centuries. To be able to recommend RSVL as a mimetic for IF

it will be important to elucidate the long-term effects of RSVL and especially the molecular pathways behind it. Further, not only the molecular mechanism but also the effects of RSVL on learning memory and mood and depression would be very interesting to investigate especially as the Thuret lab could show that intermittent fasting (IF) improves memory and increases neurogenesis. Also CR has been shown on multiple occasions to improve cognitive function (Bondolfi et al., 2004; Cohen et al., 2004; Fontan-Lozano et al., 2007; Halagappa et al., 2007; Adams et al., 2008). As described earlier RSVL acts on multiple targets and pathways and therefore: if RSVL was to be recommended as dietary intervention that has similar benefits to CR or IF further investigation is needed regarding its metabolism and its effect on cognition and aging in mammals, preferable humans.

6.3 Conclusion

In conclusion this thesis provides insight into the impact of dietary modulation of human hippocampal neurogenesis via direct effects on human hippocampal progenitor cells. The Omega-3s EPA and DHA increase survival and neurogenesis and even prevent Cortisol induced changes on cell division, neurogenesis and survival. Differences in EPA and DHA are known, however the exact mechanism remains to be uncovered. In this thesis I have shown for the first time in on a human hippocampal cell line that DHA appears to be required for neuronal maturation while EPA pre-treatment is sufficient to induce differentiation.

Figure 6-1 gives an overview based on my research on how RSVL, PPARγ, *Klotho* and intermittent fasting act on hippocampal neurogenesis. The stilbenoid RSVL increases cell division and neurogenesis but also increases apoptosis, however in the Cortisol stress model RSVL also increases cell division and neurogenesis, but does not affect apoptosis or survival. The longevity gene *Klotho*, which is increased in the hippocampus of mice maintained on an intermittent fasting diet is involved in cell fate commitment, cell division and apoptosis. Furthermore, *Klotho* expression can be increased by RSVL and RSVL partly requires PPARγ to act on Hippocampal neurogenesis. Thus within this thesis I have shown for the first time using a human hippocampal cell line that diet -here Omega-3 fatty acids and RSVL- impacts on human hippocampal neurogenesis and that the longevity gene *Klotho* plays an important role in regulating neurogenesis. Before to be able to make dietary recommendations on Omega-3 fatty acids, RSVL or fasting for prevention or treatment of cognitive decline or mood disorders, it will be necessary to conduct further investigations in animal models to be followed by human intervention studies. To uncover new

drug targets mimicking diet, more comprehensive data are needed on their molecular mode of action. Immediately, further studies should be aimed at elucidating (1) the molecular mechanisms of EPA, DHA and RSVL mediating their effect on neurogenesis as well as their differential effects on cognition; and (2) the effects of *Klotho* on AHN and behaviour in knock down and over-expression mouse models .

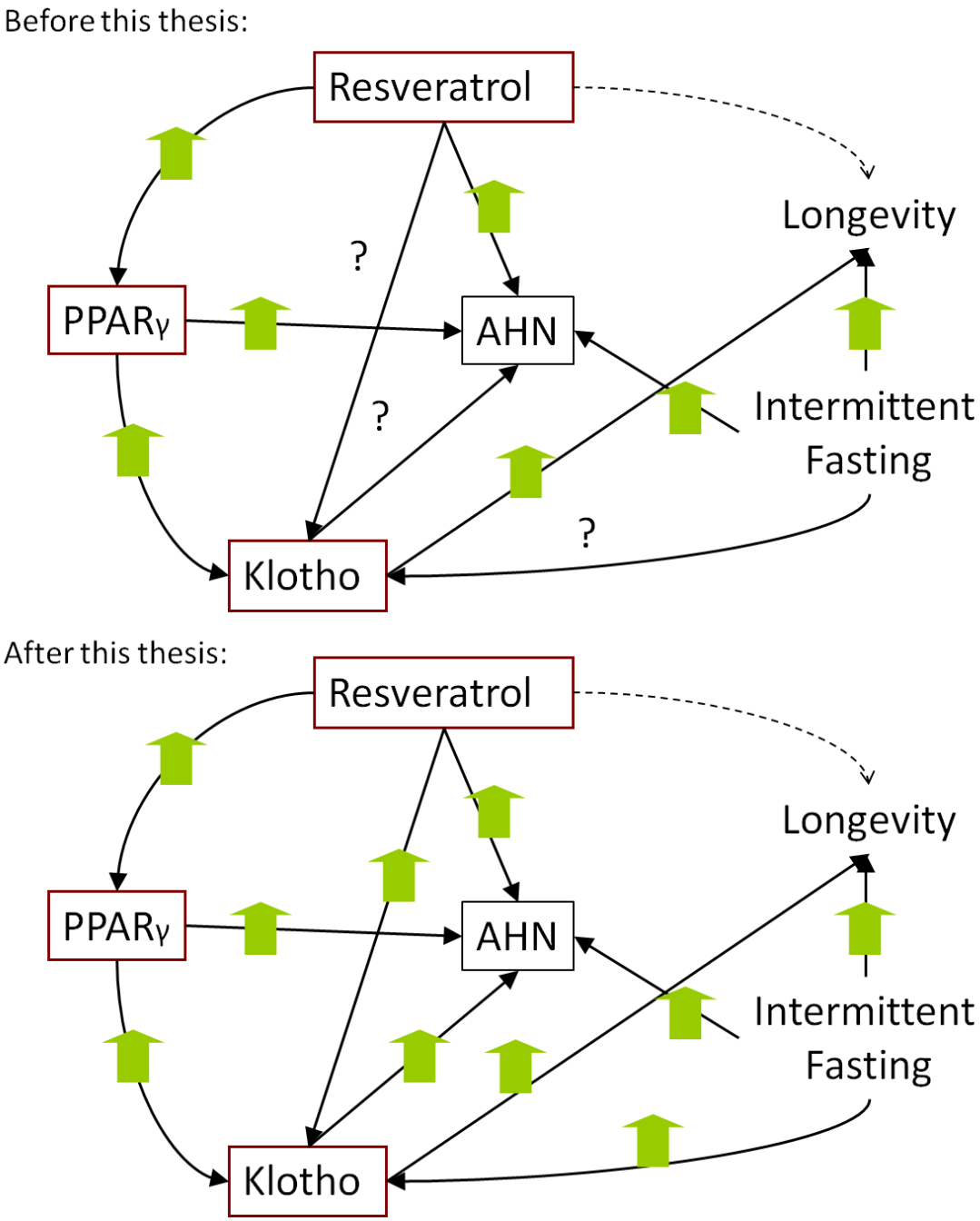


Figure 6-1 Overview of Resveratrol, PPARγ, Klotho and AHN: Before and After this thesis.
Green arrows: increased expression/activation, dotted line: in short lived organisms only

Chapter 7 Appendix

FORMULA			
Material names and concentrations:		Final conc.	Volume for 500 ml
DMEM F12 (Sigma D6421)			500.00 ml
Discard volume			10.00 ml
Human Albumin Solution 200g/l (Baxter VNA1F034/ Octapharma)	20 %	0.03%	0.75 ml
Apo-Transferrin, human (Scipac T100-5)	50mg/ml	100µg/ml	1.00 ml
Putrescine DiHCl (Sigma P5780)	8.1mg/ml	16.2µg/ml	1.00 ml
Insulin, human recombinant (Sigma I9278)	10mg/ml	5µg/ml	0.25 ml
Progesterone (Sigma P8783)	20µg/ml	60ng/ml	1.00 ml
L-glutamine (Invitrogen 25030024)	200mM	2mM	5.00 ml
Sodium Selenite (Sigma S9133)	20µg/ml	40ng/ml	1.00 ml
w GFs			
FGF2 (Peprotech 100-18B)	10µg/ml	10ng/ml	0.50 ml
hEGF (Peprotech 100-15)	10µg/ml	20ng/ml	1.00 ml
4-hydroxy-tamoxifen (4OHT) (Sigma H7904)	1µM	100nM	50 µl

Table 7-1 Ingredients for full growths media for HPC03A/07 cell line

Drug	Final concentration	Manufacturer	Solvent
α-Linolenic Acid (18:3 n-**3**) (ALA)		Sigma	100% Ethanol
Eicosapentaenoic Acid (20:5 n-**3**) (EPA)	10uM	Sigma	100% Ethanol
Docosahexaenoic Acid (22:6 n-**3**) (DHA)	10uM	Sigma	100% Ethanol
Stearidonic Acid (18:4 n-**3**) (SDA)	2uM	Sigma	100% Ethanol
Arachidonic Acid (20:4 n-**6**) (SDA)		Sigma	100% Ethanol
GW9662 Irreversible PPARγ blocker	80nM/ 1uM	Sigma	DMSO
Rosiglitazone PPARγ activator	3uM	CAYM71740-5	DMSO
Resveratrol polypheno	1uM	Sigma	100% Ethanol/ DMSO

Table 7-2 List of all tissue culture drugs used for treatment in this thesis

Protein	Cells labelled/ funciton	Antibody type	Company	Number	Dilution Tissue culture	Dilution Sections	Dilution WB
Proliferation marker							
BrdU	Dividing cells S-Phase	Rat	serotec	OBT0030	1:500		
Ki67	Dividng cells Cellcycle	Rabbit	abcam	ab15580	1:500	1:250	
Nestin	Type VI intermediate filament protein	Mouse	Chemicon	mab5326	1:1000		
SOX2	TF essential for self renewal	Rabbit	Chemicon	ab5603	1:1000	1:1000	
Neuronal marker							
Dcx	Early neurons	Rabbit	abcam	ab18723	1:1000	1:10000	
MAP2ab	Neurons	Mouse	abcam	ab11267	1:500		
NeuN	Mature neurons	Mouse	Chemicon	IHCR1001-6	1:10	1:10	
Glial marker							
GFAP	Astrocytes	Rabbit	Dako	Z033401	1:1000	1:500	
S100beta	glial cells of NS	rabbit	Dako	Z0311	1:500		
Apoptosis marker							
Cleaved Caspase-3	Activated Caspase-3, executioner of apoptosis	rabbit	Cell signalling	#9664	1:500		
Klotho							
Klotho	membrane + secreted form (hKl2)	rat 1.8mg/ml	Kyowa Hakko Kirin	KM2076	1:500		1:5000
Klotho	membrane form (hKl)	Rat 0.99mg/ml	Kyowa Hakko Kirin	KM2119	1:500		
Klotho	membrane + secreted form (hKL1+2)	mouse	R&D	MAB1819		1:500	

Table 7-3 Primary antibodies used for the human embryonic HPC03A/07 cell line, sections and WB.

AB	Host	Wave length	Company	No	Dilution		
					cells	tissue	WB
anti rat	donkey	488	Invitrogen	A21208	1:500	1:250	
anti goat	donkey	488	Invitrogen	A11055	1:500	1:250	
anti mouse	donkey	594	Invitrogen	A21203	1:500	1:250	
anti rabbit	donkey	488	Invitrogen	A21206	1:500	1:250	
anti rabbit	donkey	594	Invitrogen	A21207	1:500	1:250	
anti rat	goat	680	Invitrogen	A21098			1:3000

Table 7-4 Alexa fluor secondary antibodies used in this study

Primary antibodies	Secondary antibodies
BrdU (rat) Ki67 (rabbit)	Anti rat 488 Anti rabbit 594
Dcx (rabbit) MAP2 (mouse)	Anti rabbit 488 Anti mouse 594
KM2119 (rat) Caspase (rabbit)	Anti rat 488 Anti rabbit 594
SOX2 (rabbit) Nestin (mouse)	Anti rabbit 488 Anti mouse 594

Table 7-5 Combinations of primary and secondary antibodies used for PPARγ and Klover experiment

Primary antibodies	Secondary antibodies
BrdU (rat) Ki67 (rabbit)	Anti rat 488 Anti rabbit 594
Dcx (rabbit) MAP2 (mouse)	Anti rabbit 488 Anti mouse 594
KM2119 (rat) S100b (rabbit)	Anti rat 488 Anti rabbit 594
SOX2 (rabbit) Nestin (mouse)	Anti rabbit 488 Anti mouse 594

Table 7-6 Combinations of primary and secondary Antibodies used for the siRNA Klotho experiment

Primary antibodies	Secondary antibodies
BrdU (rat) Ki67 (rabbit)	Anti rat 488 Anti rabbit 594
Dcx (rabbit) MAP2 (mouse)	Anti rabbit 488 Anti mouse 594
Caspase (rabbit)	Anti rabbit 594
SOX2 (rabbit) Nestin (mouse)	Anti rabbit 488 Anti mouse 594

Table 7-7 Cominations of primary and secondary Antibodies used for the O3 prevention experiment

Reagent	Stock concentration	Volume to add	Final concentration
Na-Hepes pH 7.5	1000 mM	200 ul	20 mM
NaCl	5000 mM	200 ul	100 mM
Triton X100	10%	1500 ul	1%
NaF	500 mM	300 ul	15 mM
Phosphatase inhibitor	100X	100 ul	1X
Na4P2O7	100 mM	50 ul	10 mM
Na-EDTA	500 mM	20 ul	1 mM
Protease inh. Cocktail	Roche complete mini tablets	1 tablet	1X
H2O		7530 ul	

Table 7-8 Lysis Buffer for Western Blot

SDS-Page Gel	**10% gel**
ProtoGel30% (national diagnostics)	3.33 ml
4x Resolving buffer (national diagnostics)	2.50 ml
Deionised water	4.15 ml
10% APS (Sigma)	100 ul
TEMED (Sigma)	10 ul
Stacking buffer	**4% gel**
ProtoGel30% (national diagnostics)	750 ul
4x Stacking buffer (national diagnostics	1.25 ml
Deionised water	3 ml
10% APS (Sigma)	50 ul
TEMED (Sigma)	3 ul

Table 7-9 Reagents used for SDS-Page gel

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